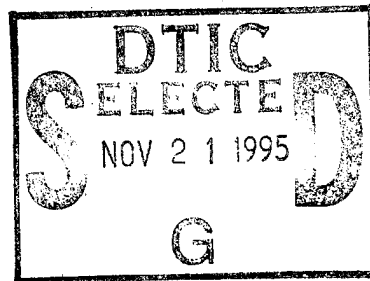AD_____

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PRINCIPAL INVESTIGATOR(S): John S. Lazo, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
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Substantial improvements in the prevention, detection and treatment of breast cancer require the addition of new laboratory-trained investigators. The objective of this predoctoral training program is to attract new investigators into the field of breast cancer research and to provide them with an interdisciplinary predoctoral training experience focused on this malignant disorder.

The Program comprises a graduate faculty of 32, who are members of 12 departments at the University of Pittsburgh and are interested in breast cancer and graduate education. An infrastructure has been developed to allow graduate students entering any of the 7 Institutional Ph.D. granting programs to be selected for membership in this Predoctoral Training Program. The Predoctoral Training Program in Breast Cancer Biology and Therapy requires a core of 32 credits of formal course work, including participation in an ethics course, a weekly seminar/research-in-progress series and a newly constructed interdepartmental course on Breast Cancer Biology and Therapy. A minimum of 72 credits with a cumulative grade point average of at least 3.0 must be obtained prior to graduation. Student retention and progress is monitored by the Breast Cancer Training Grant Executive Committee.

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FOREWORD

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D.   Table of Contents

E. INTRODUCTION

1. Background

Grants to support training of predoctoral students are usually given to a particular training program in an established scientific discipline or a subdiscipline, rather than for training in a specific disease entity or in a particular model system. Thus, training grants are relatively common in pharmacology, virology, immunology, epidemiology, psychology or biochemistry, regardless of the specific problems various investigators from these disciplines are addressing. What distinguishes these discipline-based predoctoral training programs from our **Training Program in Breast Cancer Biology and Therapy** is our multidisciplinary approach and the focus on a specific and important disease. The overall philosophy of this training proposal is to identify qualified graduate students in existing basic life science departments, to educate them in the problems in breast cancer and to enhance their research capabilities in this field. Our Training Program intends to expand the existing pool of investigators studying breast cancer. Moreover, the program is designed to encourage currently funded investigators to focus on breast cancer as an area of study; this is an important programmatic by-product because it fosters ongoing interdisciplinary research efforts by an array of well-funded investigators.

Breast cancer continues to be a leading cause of death of women in the United States. The magnitude of this disease demands novel approaches to improve our understanding of its etiology as well as methods of detection, prevention and treatment. Yet each year major medical and graduate schools, among them the University of Pittsburgh, continue to successfully graduate hundreds of Ph.D.s impeccably trained in basic principles of endocrinology, molecular genetics, psychology, neuroscience, biochemistry, cell biology, immunology, pharmacology and epidemiology, who have little appreciation for the contemporary problems and advances related to breast cancer. Most of these students have been narrowly trained in the details of their chosen basic science discipline. Very few of these recent Ph.D. graduates pursue postdoctoral training in breast cancer and fewer yet go on to establish successful research laboratories devoted to breast cancer. Part of the problem is the severe shortage of suitable mentors for these students, who could guide them in this direction. Our **Training Program in Breast Cancer Biology and Therapy** is designed to alter this situation. It encourages successful and enthusiastic investigators in various disciplines to address questions related to breast cancer in part by giving them students, who can be educated while participating in the project; the program emphasizes the multidisciplinary approach to the problem; it educates a significant number of young investigators, who would have otherwise worked on other projects, on the importance of breast cancer; it emphasizes the intrinsically interesting biological questions raised by breast cancer and the potential impact and social benefit of working on breast cancer.

F. BODY OF PROPOSAL

1. Technical Objectives

a) Recruit qualified predoctoral students to the **Training Program in Breast Cancer Biology and Therapy.**

b) Educate students in the fundamental principles of breast cancer pathobiology and therapy.

c) Evaluate the progress of the enrolled students.

d) Evaluate the program and seek additional funds and resources.

2. Training Environment

The University of Pittsburgh, founded in 1787, is one of the oldest institutions of higher education in the United States. At present it comprises 16 schools having more than 2,800 faculty and 35,000 students. Of the total student population, 9,940 are currently enrolled in Ph.D. degree programs and 1752 are in professional schools of Medicine, Law or Dentistry. There are currently 150 students pursuing Ph.D. degrees at the University of Pittsburgh SOM, 103 Ph.D. graduate students in the GSPH and 17 in the Biopsychology Program of the Department of Psychology in FAS.

The Pittsburgh Cancer Institute (PCI) was established in 1984 to strengthen and expand cancer core and educational resources in the Western Pennsylvania region by developing new, more effective approaches to the prevention, diagnosis and treatment of cancer and by enhancing professional and lay educational programs. This is especially important because the Western PA region has the oldest population of any in the US; this extends to women. Thus, the projected incidence of tumors in the female population is extremely high. In less than 10 years the PCI has become the major focal point for research and education not only in Western Pennsylvania but also Northern West Virginia and Eastern Ohio; it is now ranked 6th in the United States among recipients for NCI funding with more than $29 million annually; the PCI has dedicated basic and clinical research facilities totaling over 250,000 square feet for laboratory studies, 28,000 square feet for out-patient services and over 100 beds for cancer in-patients; over 200 women are treated annually for breast cancer at the PCI. The PCI has been responsible for recruiting more than 100 cancer researchers to the Institution, including both the Training Program Director and the Co-Director, and in 1990 the PCI was designated by the NCI as a National Comprehensive Cancer Center. Thus, there is a cohort of young and very enthusiastic investigators available for the educational and research mission of the PCI.

The PCI is a Vanguard Center for the Women's Health Initiative. In addition, Carnegie Mellon University, which is affiliated with the PCI and shares educational

programs with the University of Pittsburgh, is physically contiguous with the University of Pittsburgh. Carnegie Mellon has 7,259 student of which 1,164 are enrolled in Ph.D. programs. Thus, within a very small geographical area there is large density of students with a wide variety of interests and talents. This results in a very dynamic and exciting academic environment, which is conducive for interdisciplinary programs. Indeed one of the hallmarks of the University of Pittsburgh's campus has been the successful development of joint educational program with Carnegie Mellon University, such as the current NSF Fluorescence Center, the NSF/DoD Supercomputer Center, the Biotechnology Center and NIH supported M.D./Ph.D. program (T32-GM08208-05, Joseph M. Furman, PI, 10 positions). As one of the top fifteen recipients of NIH grants, the University of Pittsburgh Medical Center has placed particular emphasis on the importance of external research funds as a vehicle for stimulating high caliber research experience and education. Predoctoral training programs exist in all of the 5 basic medical science departments. The Dean of the SOM provides 22 (one year) predoctoral fellowships annually to these departments. Federal supported predoctoral funds are also available from the NIH Multi-Disciplinary Pulmonary Research Training Grant (2T32-HL07563-06; Robert M. Rogers, PI, 4 predoctoral positions), the NIH Pre- and Postdoctoral Training in Neuroscience (T32-18273-06; Michael J. Zigmond, PI, 7 predoctoral positions), the NIH Predoctoral Training Grant in Pharmacological Sciences (GM08424-02); J. S. Lazo, PI 3 positions) and the U.S. Department of Education Graduate Assistance in Areas of National Need Program (P200A20421; Christine Milcarek, PI, 8 positions). Thus, there is a robust environment for interdisciplinary graduate education in and around the SOM, GSPH and FAS of the University of Pittsburgh.

3. Program Director and Participating Faculty

The **Predoctoral Training Program in Breast Cancer Biology and Therapy** was formally initiated in September 1994 with the awarding of the US Army Training Grant DAMD1-94-J-4039. The Program Director is John S. Lazo, Chair of the Department of Pharmacology and Co-Director of the PCI Experimental Therapeutics Program, and the Co-Program Director is Olivera J. Finn, Associate Professor of Molecular Genetics and Biochemistry and Director of the PCI Immunology Program. Dr. Lazo has had more than 20 years of research experience in cancer biology and experimental therapeutics. Much of his early work has been directed at mechanism of drug action and drug resistance. Most of this research has been tumor type-independent in focus. He has been a member of the Board of Directors of the American Association of Cancer Research and Chair of the 1992 Gordon Research Conference on Chemotherapy of Experimental and Clinical Cancer. He is collaborating with Dr. Olivera Finn to couple antimucin antibodies to DNA cleaving agents and is examining the role of protein kinase C signalling systems in breast cancer cells. Dr. Lazo has been a Ph.D. thesis advisor or Committee Member for 16 Ph.D. candidates and has trained 21 postdoctoral fellows; he currently is thesis advisor for 1 Ph.D.

candidate, who is working on issues related to breast cancer but not funded by this Training Grant. Two of his previous postdoctoral fellows are now investigating new anticancer agents as clinical pharmacologists at a major pharmaceutical firm (Bristol Myers Beecham) and 1 is designing new diagnostic agents at a biotechnology company. Since 1976 Dr. Lazo has been intimately involved in both graduate and medical education and since 1979 he has taught a graduate level course almost every year. His basic medical science preparatory book for second year medical students published by Williams and Wilkins (*Review of USMLE Step One*) is among the most popular books of its kind (almost 50,000 copies published) and is about to enter its fourth edition. Writing and reviewing this book has given the Program Director a broad background in both basic and clinical issues related to malignancies including those associated with the breast. He is also PI of an NIH Predoctoral Training Grant in Pharmacological Sciences. The Co-Director, Dr. Olivera Finn, has been investigating breast cancer biology and immunology since 1985. She has trained 7 Ph.D. students and 7 postdoctoral fellows. Three of her Ph.D. students and 2 postdoctoral fellows have continued their research in breast cancer immunology in their new positions as postdoctoral fellows or assistant professors. She has also been a thesis committee member for 22 Ph.D. candidates. There are currently 3 graduate students in her laboratory, 2 of whom are doing research in breast cancer. In addition there are 3 postdoctoral fellows in her laboratory investigating the biology and immunology of breast cancer. In February 1993 Dr. Finn was invited to testify before the President's Cancer Panel, the Special Commission on Breast Cancer, on the future direction of breast cancer and breast cancer vaccines.

The participating faculty members have been drawn from the over 175 members of the Graduate School at the University of Pittsburgh, who are eligible to train students enrolled in a Ph.D. degree granting program. We have selected these 32 faculty members by carefully evaluating them for excellence in the following categories: extramural research support; previous educational experience; research interest in cancer, particularly breast cancer; diversity of research interest and, suitability as a mentor. We have made a special effort to include a significant number of clinically trained investigators (30% of the total faculty have an M.D. degree) to ensure the appropriate exposure of students to clinically relevant issues associated with breast cancer. Participation in this training grant is not viewed as exclusionary and new members will be considered by the Training Program Executive Committee throughout the training program funding period. Listed below (Table 1) are the members of the faculty, their departmental affiliation and a brief description of their research interest as related to breast cancer to illustrate the diversity of the faculty members and their interactions.

Table 1. Faculty of the Training Program

Faculty	Department	Major Research Interest
John S. Lazo, Ph.D., Program Director	Pharmacology	Chemotherapy, Drug resistance, Apoptosis
Olivera J. Finn, Ph.D., Program Co-Director	Mol. Genetics & Biochemistry/Surgery	Tumor Immunology, Mucins, Immunogenetics
Edward D. Ball, M.D.	Medicine	Bone marrow transplant, Cell surface markers
Andrew Baum, Ph.D.	Psychiatry/Psychology/Beh. Neuroscience	Behavioral medicine, Stress
Robert A. Branch, M.D.	Medicine	Clinical pharmacology, Drug metabolism
Anthony R. Caggiula, Ph.D.	Psychology	Behavioral immunology, Hormones
David L. Cooper, Ph.D.,M.D.	Pathology	Transcription control, Cell matrix, Gene therapy
Andrea Cortese-Hassett, Ph.D.	Pathology	Molecular genetics, Immunology
Billy W. Day, Ph.D.	Environ. & Occupat. Health/Pharm. Sci.	Molec. toxicol., Estrogen, Computational chem.
Albert D. Donnenberg, Ph.D.	Medicine	Bone marrow transplant.
Maryann A. Donovan-Peluso, Ph.D.	Pathology	Molec. genetics, Transcription
Qing-Ping Dou, Ph.D.	Pharmacology	Cell cycle control, Cyclins, Transcription
Roy A. Frye, M.D, Ph.D.	Pathology	Oncogenes, Growth factors, Molec. biology
Joseph C. Glorioso, Ph.D.	Molecular Genetics and Biochemistry	Gene therapy
Ronald H. Goldfarb, Ph.D.	Pathology/Neurosurgery	Metastasis, Invasion, Proteases
Leaf Huang, Ph.D.	Pharmacology/Molec. Genetics & Biochem.	Liposomes, Gene therapy
Candace S. Johnson, Ph.D.	Otolaryngology/ Pharmacology	Exp. therapeutics, Cytokines, Vasculature
Lewis H. Kuller, M.D., Dr.P.H.	Epidemiology	Hormone metabolism, Diet, Endocrinology
Joseph Locker, M.D.	Pathology	Molecular diagnosis, Oncogenes
Michael Lotze, M.D.	Surgery/Molec. Genetics & Biochem.	Gene therapy, Immunotherapy
Susan A. McCarthy, Ph.D.	Surgery/Molec. Genetics & Biochem.	Immunology, T cell function, Apoptosis
Kenneth McCarty, M.D., Ph.D.	Pathology	Steroid receptors, Immunohistochemistry
Edward V. Prochownik, Ph.D., M.D.	Pediatrics/Molec. Genetics & Biochem.	Oncogenes, Early response genes
Paul D. Robbins, Ph.D.	Molecular Genetics and Biochemistry	Tumor suppressor genes, Gene therapy
Guillermo G. Romero	Pharmacology	Signal transduction
Herbert Rosenkranz, Ph.D.	Environmental and Occupational Health	Computational toxin analyses
Russell D. Salter, Ph.D.	Pathology	Immunology, Cell cycle proteasomes
Martin C. Schmidt, Ph.D.	Molecular Genetics and Biochemistry	Transcription factors
Said M. Sebti, Ph.D.	Pharmacology	Signal transduction, Exp. therapeutics, Drug resistance
Jill M. Siegfried, Ph.D.	Pharmacology	Growth factors, Her2/neu oncogene, Tumor vaccines
Theresa Whiteside, Ph.D.	Pathology/Otolaryngology	Immunology, Natural killer cells
Timothy M. Wright, M.D	Medicine/Molec. Genetics & Biochemistry	Transcriptional regulation, Interferons
Jack C. Yalowich, Ph.D.	Pharmacology	Topoisomerases, Drug resistance, Exp. therapeutics

4.   Program

The study of breast cancer biology is a complex area of investigation and further understanding of this problem as well as possible solutions will emerge only through a influx of new investigators and a combined effort of investigators from many different disciplines of modern biology and science. The overall objective of our **Training Program in Breast Cancer Biology and Therapy** at the University of Pittsburgh is to exploit the well recognized expertise of selected faculty in the areas of Endocrinology, Pharmacology, Psychology, Behavioral Medicine, Molecular Genetics, Immunology, Cell Biology and Epidemiology and their specific interests in breast cancer. Recruitment of these investigators from their parent departments into this training program is designed to support their interests in breast cancer and to provide them with an opportunity to recruit and to train young investigators in the basic principles of their discipline using breast cancer as a specific model system.

4.1   Predoctoral Training Pool

There are nine (9) Ph.D. granting programs at the University of Pittsburgh, School of Medicine: Bioengineering (Joint Program between the Schools of Engineering and Medicine), Biochemistry (Granted by the Department of Molecular Genetics and Biochemistry), Microbiology (Granted by the Department of Molecular Genetics and Biochemistry), Pharmacology, Pathology, Cell Biology and Physiology, and Neurobiology. Faculty of the **Training Program in Breast Cancer Biology and Therapy** are members of 5 of these programs. Additional faculty are from 1 Ph.D. granting program in GSPH, Environmental and Occupational Health, and 1 Ph.D. granting program from the FAS, Biopsychology. Admission to the training grant program requires a bachelor's degree with a major in chemistry, biology, physics, psychology, microbiology, biology or molecular biology from an accredited college or University, with a minimum grade point average (GPA) of 3.0. In addition, general and advanced subjects of tests of the Graduate Examination Record (GRE) must be taken.

4.2   Program Administrative Structure

The administrative structure of the Training Program uses the resources of existing programs and is co-chaired by John S. Lazo and Olivera J. Finn. The routine duties such as corresponding with potential applicants, monitoring student progress, ensuring appropriate student records, monitoring student progress, dissemination of information to faculty and student is performed by Bonnie Schiavone, Administrator of the Graduate Program, Department of Pharmacology. Seminar announcements and journal club schedules has been by Ms. Schiavone. Ms. Schiavone meets on a regular basis with the Training Program Executive Committee to evaluate student progress and program needs.

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4.3 Student Recruitment and Admission

The recruitment process began in the Spring of 1994 with an effort to identify highly qualified students, who had not yet chosen their research topic or advisor (2nd year students) or who have recently identified a breast cancer related research project (3rd year student). All faculty participating in this training program received a letter informing them of the program and announcements were posted throughout the University. Particular effort will be made to identify and encourage women and individuals from under represented ethnic groups to apply for these fellowship monies.

Applications for admission into the **Training Program in Breast Cancer Biology and Therapy** were evaluated on June 27, 1994 by the Breast Cancer Training Grant Executive Committee. This committee comprise the Director and the Co-Director of the program, Drs. Lazo and Finn, and five other faculty members selected for their research interests and diversities. These are: Drs. Kuller, Caggiula, Siegfried, McCarty and Whiteside. We received 12 completed applications and each application was discussed in great detail (Table 2). Several faculty members had interviewed the candidates and provided information about the applicant, in some cases acting as a formal advocate for the applicant. Each faculty member had one vote and admission was determined based upon total votes awarded each applicant. Applicants were judged based on their undergraduate record, results of GRE scores, performance in first and/or second year of graduate school, faculty comments, and a brief written statement of their research interest as related to breast cancer. A effort was made to ensure equitable distribution of fellowships among the represented disciplines and areas of research. The six finalists are listed below (Table 3). All awardees were notified within two weeks and no student declined the award. The Executive Committee decided the make a commitment of two years for each student pending successful completion of the first year because this would allow the student security of funding and a more meaningful graduate experience.

4.4 Course of Study

A minimum of 32 credits of formal course work and 40 credits of dissertation research are required to earn a Ph.D. in all of the participating departments. The Executive Committee has examined the progress and course grades of the students to ensure they fulfill the requirements of the Program. The students are also required to complete an Ethics course offered by University and to attend the weekly conference on Breast Cancer Biology held by Dr. K. McCarty, a member of the Executive Committee of this Program. This informal interactive working group was formulated last year and highlighted only local faculty speakers some of whom are faculty members of this program. In the next year, the seminar series will include outside speakers, who are experts in various aspects of breast cancer biology and therapy. It will also include individuals speaking on behavioral aspects of breast cancer biology and therapy.

Table 2

Applicants	Departments
Ronna Campbell	Pathology
Albert Cunningham	Environmental & Environmental Health
Raymond Ganster	Molecular Genetics and Biochemistry
Theresa Hartsell	Neurology and Pharmacology
Jie-Gen Jiang	Pathology
David Krisky	Molecular Genetics and Biochemistry
Edwina Lerner	Pharmacology
Donald Schwartz	Pharmacology
Laura Sheehan	Molecular Genetics and Biochemistry
Ernst ter Haar	Graduate School of Public Health
Steve Walker	Molecular Genetics and Biochemistry
Mark Whitmore	Pharmacology
Diane Zeleski	Pharmacology

Table 3

Student	Year	Mentor	Title of Project
Ronna Campbell	3	Michael T. Lotze, M.D.	The role of interleukin-12 in breast cancer biology
Albert Cunningham	3	Herbert S. Rosenkranz, Ph.D.	Structure-activity relationship analyses of xenoestrogens with respect to their role in carcinogenesis
Raymond Ganster	3	Martin C. Schmidt, Ph.D.	Investigation of STD1 function in the cell cycle regulation of SUC2 gene expression
David Krisky	3	Joseph C. Glorioso, Ph.D.	The development and use of Herpes Simplex virus type 1 as a vector for anti-cancer gene therapy
Edwina C. Lerner	3	Said M. Sebti, Ph.D.	Ras/Raf interactions in breast carcinoma: inhibition of plasma membrane localization by Ras-CAAX peptidomimetics
Diane Zeleski	5	Guillermo G. Romero, Ph.D.	Calculated reduction in IRS-1 protein levels can reverse the tumorigenicity of breast cancer

The formal course work requirement for most of the departments is similar and is structured around the 4 core courses of (a) Biochemistry, Macromolecules and Metabolism, (b) Cell Structure and Function, c) Molecular Genetics and (d) Signal Transduction. The minimum course work for members of the graduate program in biopsychology are (a) Principles of Behavior, (b) Research Methods, c) Systems Neuroscience and (d) Mammalian Physiology. The second year consists of elective courses. Students supported by the **Training Program in Breast Cancer Biology and Therapy** will be required to take an additional course in breast tumor biology and therapy, which will be offered in the Spring of 1996 and taught by a number of program faculty. This course will be entitled Breast Cancer Pathobiology and Therapy. An anticipated course outline is seen in Table 4.

Table 4. Course Outline for Breast Cancer, Pathobiology and Therapy

Lecture Block	Block Organizer
Breast Biology (4 sessions)	**K. McCarty**
Topics: Normal Development	K. McCarty
Abnormal Development	K. McCarty
Molecular Genetics and Markers (4 sessions)	**D. Cooper**
Topics: Growth Factors and Signalling	J. Siegfried
Oncogenes	E. Prochownik
Suppressor Genes	D. Cooper
Cytogenetics and Molecular Genetics	J. Locker
Invasion and Metastases (2 sessions)	**R. Goldfarb**
Topics: Extracellular Matrix	D. Cooper
Proteolytic Enzymes	R. Goldfarb
Drug Therapy/Resistance (6 sessions)	**J.S. Lazo**
Topics: Principles of Chemotherapy	S. Sebti
Pharmacokinetics and Chemotherapy	J. Yalowich
Cell Cycle Checkpoints	Q. Dou
Apoptosis	J.S. Lazo
Drug Resistance	J. Yalowich
Angiogenesis and Tumor Vasculature	C. Johnson
Immunobiology and Immunotherapy (6 sessions)	**O. Finn**
Topics: Tumor Antigens	O. Finn
T and B Cell Function	S. McCarthy
Natural Killer Cells	T. Whiteside
Vaccines	O. Finn
Adoptive Therapy	M. Lotze
Gene Therapy	M. Lotze
Epidemiology and Prevention (4 sessions)	**L. Kuller**
Topics: Approaches of Risk Factor Assessment	L. Kuller
Data Acquisition and Analyses	K. McCarty
Dietary Control	L. Kuller
Environmental Toxins	B. Day
Behavior (4 sessions)	**A. Caggiula**
Topics: Stress, Immunology and Hormones	A. Caggiula
Psychological Impact of Screening	A. Baum
Promoting Compliance	A. Baum
Behavior and Therapeutic Response	A. Caggiula

4.5   *Student Research*

A brief summary of the student progress is listed below. One peer-reviewed manuscript has been published, five are in press and one is submitted. Submitted and published manuscripts are found in the Appendix.

Ronna Campbell

The role of interleukin-12 in breast cancer biology

Interleukin 12 is a heterodimeric cytokine which was purified from an Epstein-Barr virus transformed lymphoblastoid cells line based on its ability to stimulate IFN-g production, activate NK cells and act as a growth factor for T and NK cells. In addition to its immunostimulatory activities IL-12 has recently been shown to possess an antiangiogenic capacity. This antiangiogenic capacity is mediated by IFN-g which is a powerful stimulator of inducible protein-10. IL-12 is produced predominantly by phagocytic cells, including macrophages and polymorphonuclear cells but has recently been shown to be produced by epidermoid carcinoma cell lines and normal human keratinocytes. Interleukin 10 inhibits lymphocyte cytokine production, particularly IFN-g from T and NK cells, and inhibits proliferation of T cells indirectly by suppressing monocyte/macrophage production of IL-12. IL-10 is produced by various cell types including keratinocytes, monocytes and B and T cells. We hypothesize that human breast cancer cell lines, like other epidermoid cell lines, may produce IL-12 and that this may inhibit tumor growth by inducing both nonspecific immunostimulatory activities and angiogenic inhibition. We intend to test this hypothesis using various human breast cancer cell lines such as BT-20, MDA-NB-435s, ZR-75-30 and others under various culture conditions with and without stimulation. We will measure IL-12 production with ELISA and determine biological activity based on its ability to induce IFN-g production from human PBLs. We further hypothesize that the IL-12 production may be modulated by IL-10. We will test this hypothesis by adding exogenous IL-10 to cultured breast cancer cell lines and determine the effect on IL-12 production. We will also use neutralizing IL-10 antibody to determine if endogenously produced IL-10 has an effect on IL-12 production. We have already shown that EBV-transformed cell lines produce variable amounts of IL-12 and that this production is modulated by addition of exogenous IL-10.

Albert Cunningham

Structure-activity relationship analyses of xenoestrogens with respect to their role in carcinogenesis

The objective of my research into the etiology of breast cancer is to help determine whether a causative link exists between environmental estrogen (xenoestrogen) exposure and breast cancer. Since, almost by definition, environmental exposure to xenobiotics occurs on a minute scale (e.g. low chronic doses), a statistically significant relationship between exposure and carcinogenesis is often hard, if not impossible to establish. Furthermore, estrogen induced cancer is believed to occur through a "non-genotoxic" receptor-based mechanism. Although a non-genotoxic route to carcinogenesis is not disputed, the exact mechanism(s) remains elusive. The problem then is (a) to define the mechanism of carcinogenicity of estrogens in humans, (b) to determine if xenoestrogens are included in this archetype and © to determine whether it requires the development of a new mechanism-based risk assessment paradigm. The use of mechanistic-based information in defining carcinogenic risk to humans has gained acceptance recently.

At the time that I submitted my proposal for this fellowship I was concluding a structure-activity relationship (SAR) study of the carcinogenicity of diethylstilbestrol (DES), a potent human estrogenic carcinogen. The study was conducted with the aid of MULTICASE, a SAR expert system, and META, an expert system designed to predict human xenobiotic biotransformation products.

This study has yielded insight into the non-genotoxic nature of estrogen-related cancer since neither DES nor its metabolites are potential mutagens. Moreover, the analyses suggested (a) that the observed carcinogenic spectrum of DES reflects the activity of metabolic intermediates and (b) that the carcinogenicity of DES in mice is due to the presence of a 6Å geometric descriptor that could be related to an estrogen receptor. In a further study, this distance descriptor biophore has been found to be present in some, but not all, estrogens and antiestrogens. Thus a dichotomy exists between estrogens with and without the 6Å biophore. This dissimilarity may prove useful in separating carcinogenicity from estrogenicity if the two are separate phenomena.

A similar SAR investigation was conducted into the possible human carcinogenicity of tamoxifen (TMX) and toremifene (TRM), two potent antiestrogenic compounds in human breast tissue. Although the carcinogenicity of these compounds has not been established in humans, these chemicals are relevant to human carcinogenesis due to their similarities with DES. Also, TMX has been implicated in the induction of rare endometrial cancer in humans as well as the induction of hepatocellular carcinomas and hepatic adenomas in experimental animals.

TRM has been shown to be non-carcinogenic to rodents under conditions in which TMX induces liver tumors in rats. Thus, comparison of TMX and TRM could permit a determination of the mechanism of carcinogenicity of this group of chemicals, especially with respect to the possibility that their estrogenicity and carcinogenicity are separate phenomena.

TMX, TRM and the majority of their metabolites are predicted to be non-mutagenic in *Salmonella*. However, the possibility exists that metabolism of the putative desmethyl intermediates may yield hydroxylamines capable of modifying the deoxycytidine and deoxyguanosine moieties in DNA. Thus, TMX and TRM are potentially genotoxic. This is supported by the finding that the major DNA-adduct in TMX-treated rats involves deoxyguanosine and deoxycytidine.

Additionally, TMX, TRM and many of their metabolites are predicted to be rodent carcinogens. Moreover, many of these metabolites contain a 6Å or 8.4Å distance descriptor biophore. The 6Å biophore identified in these compounds is the same biophore indicative of carcinogenesis of DES. The prediction of the carcinogenicity of TRM is not in accord with studies published thus far. However, the reports available have not excluded this possibility since the protocols used have not addressed it systematically.

The main impetus of work to this point has been in the elucidation of structural features of estrogenic chemicals as they relate to carcinogenesis. The benefit of using pharmaceutical estrogens has been that their mechanism of action is better understood than xenoestrogens and the doses received by patients is much higher than environmental exposures, thus the carcinogenic response, if it exists, should be easier to observe.

At the present time the focus of my investigations is a SAR analysis of the large carcinogenic potency database (CPDB) of Lois Gold et al. The CPDB is a compilation of the carcinogenicity of 1136 chemicals from 4487 experiments. Three rodent subsets have been extracted from this database (i.e. rodent, mouse and rat). A manuscript describing the structural features of the mouse database will be completed shortly followed by the rat and rodent reports. The rat and rodent analyses should be concluded rather quickly since the groundwork for the investigation was laid during the mouse analysis.

In the near future I will be assembling a database on the estrogenicity of chemicals. With this database I hope to identify structural feature of chemicals indicative of there estrogenicity. This will be a more inclusive investigation than the identification of the previously discussed 6Å biophore. Structural features associated with estrogenicity can then be compared to features related to carcinogenicity. This comparison may reveal if carcinogenicity and estrogenicity are separate phenomena or are intimately related. The mechanism(s) of action of estrogens and xenoestrogens may be further defined. Additionally, structural information about estrogens may help determine whether xenoestrogen exposure is specifically related to the induction of human breast cancer.

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Raymond Ganster

**Investigation of STD1 function in the cell cycle regulation of *SUC2*
gene expression**

STD1 was originally isolated as a high copy number suppressor of a dominant negative mutation in the yeast TATA Binding Protein (TBP). Increased *STD1* gene dosage rescues the gene specific transcriptional defects and partially relieves the growth defect caused by overexpression of TBPΔ57 which expresses the evolutionarily conserved carboxy-terminal "core" domain of TBP while lacking 57 amino terminal residues (see reference 1). *MSN3* (*STD1*)was independently cloned in the Carlson lab as a multicopy suppressor of the growth defect of *snf4* mutant yeast strains when growing on raffinose as a carbon source. High copy number *STD1* also rescued the *snf4* defect in derepression of the *SUC2* gene in response to glucose starvation. Furthermore, high copy number *STD1* caused the derepression of *SUC2* in a wild type cell growing in the presence of glucose. *SUC2* encodes the yeast sucrose invertase which is transcriptionally repressed by

chromatin in cells growing in the presence of glucose. However, the derepression of *SUC2* occurs in response to glucose limitation and is required for growth on alternative carbon sources. Significantly, *STD1* and a functional homologue *MTH1*, when deleted together in a *std1⁻mth⁻* double knockout strain impair the derepression of *SUC2*, while inhibiting growth upon alternative carbon sources. *STD1* gene dosage effects occur at the level of *SUC2* transcription. *snf4* encodes a protein which interacts with the evolutionarily conserved SNF1 kinase and is required for full SNF1 kinase function and *SUC2* derepression. Furthermore, *snf1* is required for *SUC2* derepression and derepression by high copy number *STD1*. Interestingly, STD1 can interact with SNF1 and TBP in the yeast two-hybrid system and in column binding assays. The interaction with TBP is direct and has functional consequences for *SUC2* derepression (see reference 2). Furthermore, recent unpublished data indicate that both high copy number STD1 or TBP can suppress the growth defects of *snf 4* or *std1-mth1* null allele strains growing upon alternative carbon sources.

A wide assortment of transcriptional regulators have been isolated in genetic screens which include repressors (i.e. MIG1, TUP1/SSN6, SKO1, histones and HMG-like proteins) and activators (SWI/SNF complex, MSN2, STD1 (MSN3), and MSN4) of *SUC2* expression. However, scant information exists regarding the *cis-trans* sequence requirements and/or protein interactions through which these factors exert transcriptional regulation. For instance, while others have described SWI/SNF interactions with nucleosomes in genetic screens and biochemical assays, we have recently described the "naked DNA" binding properties of a highly purified SWI/SNF complex (see reference 3). The evolutionarily conserved SWI/SNF complex contains at least 11 proteins (including SWI1, 3 and SNF2, 5 and 6) and regulates the transcription of a broad spectrum of genes. Interestingly, human*SNF2* (*hSNF2*) isolated the Rb tumor suppressor gene while serving as a bait in a yeast two-hybrid system screen. hSNF2 is a member of the human SWI/SNF protein complex (see reference 3) and cooperated with Rb in suppressing the growth and transformed phenotype of some tumor cell lines while implicating a role for hSNF2 in the G1 phase of the cell cycle. Furthermore, recent data show that high copy number *STD1* or *TBP* dramatically reduces the growth of a *snf 5* strain when growing upon alternative carbon sources. The human *SNF5* was recently cloned in a yeast two hybrid system screen using the HIV integrase as a bait and was seen to facilitate HIV integration *in vitro*. This could serve to place HIV under the control of SWI/SNF regulation, which could determine whether HIV remains latent or rather, actively replicates in dividing cells. As is the case with *SNF1*, *SNF5* is required for the high copy number *STD1* derepression of *SUC2*. The relationship of STD1 and TBP to the SWI/SNF complex is under investigation.

*STD1*gene dosage effects upon RNA expression have also been observed for *ADH2*, *CUP1*, and for the repressed and "induced" expression of *INO1*. However, the precise nature of the regulatory signal(s) and predictable *STD1* dependent effects have remained elusive. Possible explanations for the inconsistency in *STD1* gene dosage effects at these

loci include that these experiments were performed at different times over a several year period and that the yeast strains, media carbon source, and densities of the cell cultures often varied, precluding direct comparisons amongst the data. This is in contrast to the predictable *STD1* dosage effects upon *SUC2* derepression in the presence or absence of glucose. Recent experiments analyzing *STD1* gene dosage effects upon *SUC2* RNA expression in response to fresh glucose, .05% glucose, or NO glucose treatments indicate the following. The derepression of *SUC2* caused by high copy number *STD1* occurs with similar kinetics to the normal de-repression of *SUC2* in response to glucose limitation (.05% glucose). Furthermore, strains deleted for STD1 are altered in the response to a total glucose starvation (NO glucose). Specifically, *SUC2* derepression is not observed in wild type cultures which are abruptly starved of glucose (NO glucose) however, some derepression of *SUC2* is observed in an isogenic *std1-mth1-* double deletion strain. Interestingly, gradual glucose starvation results in a *SNF1* dependent growth arrest in late G1 coupled with *SUC2* derepression, while abrupt glucose starvation results in an immediate asynchronous arrest with decreased *SUC2* derepression. This implies that cell cycle progression or passage of cells through a specific cell cycle point may be required for efficient *SUC2* derepression and that the glucose signalling mechanism may input to the cell cycle machinery. Indeed, preliminary evidence in synchronized cells demonstrates that the *SUC2* mRNA accumulates in a cyclical fashion and that high copy number *STD1* alters the amplitude and phase of *SUC2* mRNA accumulation. One interpretation of the above result (that the STD1 deletion strain can partially derepress *SUC2* expression after an abrupt glucose starvation, whereas a wild type strain does not) is that a *std1*-deletion strain has a defect in the *SNF1* dependent G1 growth arrest, and that the resulting cell cycle progression permits some *SUC2* derepression. In this regard, *STD1* is also required for efficient sporulation/meiosis of yeast diploid strains, an event which is coupled to arrest in late G1. Furthermore, *STD1* promoter DNA contains putative SWI4 cell cycle regulatory elements. To investigate glucose and/or *STD1* dependent cell cycle input to the *SUC2* derepression pathway, analyses of *SUC2* RNA are now being performed using asynchronous and cell cycle synchronized cultures of isogenic strains that contain 0, 1, or high copy number *STD1* gene dosage under a variety of media carbon source conditions. Furthermore, the expression of *STD1* mRNA and protein will be analyzed under identical conditions.

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David M. Krisky

The Development and Use of Herpes Simplex Virus Type 1 as a Vector for Anti-Cancer Gene Therapy

There are many forms of transfer available for the addition of genetic material to cellular targets. Today, most approved gene therapy protocols use adenoviral or retroviral virus based vectors or a number of non-viral mechanisms, such as liposomes, for transfer of DNA. There are sizable inherent problems with each of these systems. Adenoviral vectors show transient expression, high immunogenicity, low capacity, and troublesome wild-type revertants. Retroviral vectors produce low titers of viral stock which require treatment with retrovirus producing cell lines, which is undesirable due to problems of genetic stability . Liposomes and/or naked DNA administration have been seen to be transient expressors as well as inefficient vectors compared to their viral counterparts. Due to these problems and others, viral vectors such as Herpes Simplex Type 1 (HSV-1) and Adeno-associated virus (AAV) have gained popularity as possible gene therapy vectors. HSV-1 is a large, 152kB, double stranded DNA virus which has been well characterized. Deletion mutants in essential genes, such as ICP4, have been well characterized and show low rates of revertants while retaining the ability to be grown to high titers. HSV-1 as a viral vector is not without problems. It too suffers from transient expression and compared to other viral vectors is difficult to genetically modify although it does allow for generation of high titer stocks.

This project is involved in the development of HSV-1 as a gene therapy vector for possible anti-cancer uses. To date a number of replication deficient viruses have been developed and tested in rat and mouse models. All of these viruses are based upon the replication deficient ICP4 deletion mutant d120 (DeLuca et al., 1985). In each case an expression cassette was recombined in the thymidine kinase locus of the virus. Each cassette contains beta-galactosidase driven by the HCMV immediate early promoter as well as a therapeutic gene driven by the HSV-1 strong ICP4 immediate early promoter . The therapeutic genes inserted in this class of viruses are HSV-TK, hIFN-g, hIL-2, mIL-4,

and mB7.1. The most thoroughly tested virus from this group has been the HSV-TK producing virus. HSV- TK is one of a class of suicide genes which can cause cell death when used in conjunction with the anti-herpetic drugs acyclovir or ganciclovir. In the presence of HSV-TK these drugs are chemically modified to become defective nucleotides which can block DNA synthesis via chain termination and lead to death in dividing cells. The effect of these nucleotides can extend beyond the cells which are infected. Numerous studies have shown that these defective nucleotides are exported to surrounding cells via gap junctions which can lead to subsequent killing termed the "bystander effect" (Freeman et al., 1993). In vitro studies involving the 9L rat gliosarcoma has shown the development of a bystander effect upon treatment with ganciclovir which leads to greater than 85% killing with an infection rate less than 10% of cells (moi=0.1) and complete killing at higher infection levels (moi=1 or 10) . This mutant virus also shows a high level of cytotoxicity without the addition of ganciclovir. At an moi=1 approximately 75% of 9L cells are killed without the addition of ganciclovir (GCV), this level increases to about 90% at an moi=10.

This HSV-TK d120 mutant has also been tested in a the 9L gliosarcoma model in rat brains and MCA207 fibrosarcoma and the p815 mastocytoma line in subcutaneous models in mice. For the 9L rat gliosarcoma model 180-220g male rats are given an intracranial stereotactic injection of 4 x104 9L cells on day zero. Viral treatments are given in the form of stereotactic injections of 3 x 107 plaque forming units in a 9 point cubical 1mm x 1mm x 1mm area around the tumor injection site. The average survival times (n=5) for animals with treatment beginning on day 7 are as follows; control 17.6 days, control + GCV (4 days at 30/mg/kg/day) 16.6 days, virus alone 21.6 days , virus + GCV (4 days at 30mg/kg/day) 18.6 days. The average survival times (n=5) for animals with treatment beginning on days three improved to; control 19.2 days, control + GCV (4 days at 30mg/kg/day) 20.8, virus alone 22.4 days, virus + GCV (4 days at 30/mg/kg/day) 23.6 days. Overall these experiments suggested that the viral treatment had little effects on overall survival times in these models. In order to better understand the shortcomings of this treatment experiments were performed as above for seven days tumors but the animals were sacrificed two days post virus injection in order to view tumor histology/damage and for beta-galactosidase staining in order to measure viral spread. In virus treated animals major areas of necrosis could be seen in and around the tumor but little evidence of beta-galactosidase could be found. Associated with viral treatments were substantial areas of mononuclear infiltrates, these infiltrates have not been seen with the injection of virus alone into the frontal lobe. There was little evidence at this time of increased tumor damage due to the addition of GCV. This information when coupled with the in vitro studies of this virus suggests that the toxicity of the virus leads to death of most of the tumor cells before the TK-GCV associated killing could start. Given the wider range of killing associated with a HSV-TK/GCV bystander this cytotoxicity was in essence short circuiting a system for wider and more efficient tumor killing.

In order to improve on these results we have begun a series of modifications to our HSV-1 based vectors. Our first aim is to reduce the cytotoxicity associated with our current virus. When have begun to systematically delete the remaining immediate early genes as well as genes coding for toxic products which are packaged within the virion, such as the virion host shut off protein and VP16. Our ultimate goal is to produce a base vector which upon infection will not transport toxic virion proteins or make extraneous viral proteins which could alter cellular function. In place of these genes we have begun inserting genes which could possibly augment the HSV-TK/GCV killing as well its concomitant inflammation. Genes under consideration for this purpose include IL-2, IFN-g, GM-CSF, B-7.1, and TNF-a either alone or in combination with each other. Thus, while we construct less toxic and safer vectors at the same time we can use the large genomic size of HSV-1 to our advantage in creating multigene viruses which in both cases my lead to greater efficacy.

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Edwina C. Lerner

Ras/Raf interactions in breast carcinoma: inhibition of plasma membrane localization by Ras-CAAX peptidomimetics

For the past year (September 1, 1994 - present), I have been working in Dr. Said Sebti's lab on the mechanism by which farnesyltransferase (FTase) inhibitors disrupt Ras/Raf interaction and Ras signaling in whole cells. Our efforts in this area have resulted in the first direct evidence that non-farnesylated cytosolic Ras can act as a dominant negative inhibitor of Ras signaling and that K-Ras4B, the most abundant mutated Ras in human cancers, is geranylgeranylated. These two major findings have a great impact on future research directions in the field of targeting Ras in cancer chemotherapy. This work resulted in two *J. Biol. Chem.* publications that are attached. A brief summary of the results is described below.

The Ras oncogene plays a key role in normal cellular proliferation and differentiation and is the most frequently identified oncogene in human cancers. The role of Ras genes in human mammary carcinogenesis remains undefined (ref. 1). However, several established human breast cancer cell lines have been shown to contain a mutationally activated *ras* oncogene (ref. 2), and 60-70% of hyperplastic lesions and primary breast carcinomas are highly expressive for Ras as compared to normal breast epithelia (ref. 1). Ras is a small guanine nucleotide binding protein that cycles between its inactive GDP-bound state and its active GTP-bound state. Farnesylation and subsequent membrane association of Ras is required for its biological function and its oncogenic activity. Thus, inhibition of Ras membrane association provides a key target for novel approaches to anticancer therapy.

We hypothesized that when GTP-locked Ras accumulates in the cytosol as a consequence of inhibition of membrane association, it would bind tightly to Raf preventing it from localizing to the plasma membrane where it must be present to be activated. We sought to determine the mechanism by which inhibition of Ras processing disrupts Ras downstream signaling by determining what happens to Raf when Ras is removed from the membrane upon treating cells with farnesyltransferase inhibitors. The results from this study are documented in paper #1 (see below). Briefly, cells transformed with a GTP-locked Ras were treated with a highly potent Ras CAAX peptidomimetic FTI-277. Ras was inhibited from going to the plasma membrane and therefore remained in the cytosol where it soaked up Raf which was also inhibited from going to the membrane where it would be activated. Thus, it appears that GTP-locked Ras when accumulated in the cytosol is acting as a dominant negative inhibitor resulting in the formation of inactive Ras/Raf complexes, thereby further inhibiting the activation of Ras downstream signaling events, such as MAP kinase (refer to paper #1). These studies provided a potential mechanism by which FTase inhibitors are selective to Ras-transformed tumors over normal cells that do not have a GTP-locked Ras and therefore may not soak up Raf.

The Ras CAAX peptidomimetics are inhibitors which show great selectivity for the enzyme farnesyltransferase over a closely related enzyme geranylgeranyltransferase I *in vitro*, and they selectively block the processing of farnesylated but not geranylgeranylated proteins in whole cells. However, mammalian cells expresses four types of Ras proteins (H-, N-, KA-, and KB-Ras) among which K-Ras4B is the most frequently mutated form of Ras in human cancers. Work by our lab and others has demonstrated the inhibition of H-Ras processing and signaling, but this had not yet been shown with K-Ras4B (paper #1 and #2). We were the first to demonstrate the inhibition of K-Ras processing and signaling in whole cells with the highly potent farnesyltransferase inhibitor FTI-277. The inhibition of K-Ras processing, however, occurred at much higher concentrations than needed to inhibit H-Ras processing (see paper #1) and which were more closely equivalent to concentrations which inhibited the geranylgeranylated protein Rap1A. We, therefore, set out to determine whether a GGTase I-selective inhibitor would disrupt K-Ras4B processing and signaling. Our results demonstrate that oncogenic K-Ras4B processing

and constitutive activation of MAP kinase are potently inhibited by a GGTase I-selective inhibitor (GGTI-286) but are resistant to one selective for FTase (FTI-277) (see paper #2). The results presented in this study are critical to the future development of inhibitors of Ras prenylation as potential anticancer agents. This is a key finding since K-Ras4B is the most frequently identified mutated Ras in human cancers and its function has been resistant to FTase inhibitors.

The above studies were carried out in NIH 3T3 cells transfected by various Ras oncogenes and serve as a great model for future studies in human tumors such as the breast. Our plan is to continue to investigate Ras/Raf interaction as a target for designing more selective anticancer drugs. This will be facilitated by the newly published X-ray structure of the Ras/Raf binding domain. Furthermore, since we now have a model to detect Ras/Raf association, we will test the effects of Ras CAAX peptidomimetics and Ras/Raf association in breast cancer cell lines which have either an activated GTP-locked Ras or an overexpressed Ras to determine the efficacy of these compounds in a breast cancer model.

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Diane M. Zeleski

Insulin Receptor Substrate 1 (IRS-1) Expression and Function in Human Breast Cancer Cell Lines

Background and Significance:

My primary hypothesis is: A calculated reduction in IRS-1 protein levels can reverse the tumorigenicity of breast cancer.

The insulin receptor (IR) is a member of the tyrosine kinase growth factor receptor family. Receptor tryrosine kinases play a key role in both normal and neoplastic cell growth. IR over-expression induces a ligand-dependent transformed phenotype. A six-fold overexpression of structurally and functionally normal IR content has been reported in human breast cancer specimens as well as a number of cultured breast cancer cell lines. Additionally, IR content has been found to correlate with other clinical parameters, including tumor size and grade, that reflect increased tumor aggressiveness. Since the receptor number has been established to be aberrant, the possibility exists that by short-circuiting the signal transduction pathway just proximal to the receptor, a reversal of the tumorigenicity of these breast cancer cell lines may be realized.

A potential candidate that fulfills the aforementioned criteria is IRS-1. Numerous studies have reported that IRS-1 is an important phosphoprotein central to many of insulin's and IGF-1's signaling pathways, and it functions just proximal to the receptor. Fortunately, the rat, mouse, and human IRS-1 sequences have recently been cloned. The three protein sequences are highly conserved (>90%), particularly in their potential phosphorylation sites. This high degree of conservation across species supports the notion of the central importance of IRS-1 in both insulin and IGF-1 signaling. IRS-1 contains at least a dozen tyrosine residues and nearly 50 serine/threonine residues as potential phosphorylation sites. Although the in vivo phosphorylation status of IRS-1 has not been thoroughly characterized, the enormous number of such sites implies that it is a highly regulated phosphoprotein in downstream modulation of growth factor activation. Immediately following insulin/IGF-1 stimulation, IRS-1 is rapidly phosphorylated on tyrosine residues. Phosphotyrosinated IRS-1 is known to interact with the SH2 domains of several critical signaling proteins. These include: the regulatory 85 kD a subunit of PI3K; the tyrosine phosphatase, SHPTP2; the adaptor protein, Grb-2, essential for p21ras activation; and oncogenic adaptor proteins, such as Nck. An observations of considerable importance is the fact that the functional expression of IRS-1 protein has recently been established as an absolutely essential component for the mitogenic response in a CHO cell line. Given the fact that both insulin and IGF-1 stimulate proliferation of breast cancer cells and IRS-1 is a central component of this signaling, the study of the role of IRS-1 in the pathobiology of breast cancer is of considerable interest.

Preliminary Results:

The candidate is currently performing thesis research in the laboratory of Professor Guillermo Romero in the Department of Pharmacology. Over the past year of support on this grant, preliminary studies have focused on IRS-1 knockout experiments utilizing antisense strategies in a murine NIH3T3-derived fibroblast cell line, F442A. Antisense constructs have been generated in the established eukaryotic expression vector, pcDNA3 (In Vitrogen), carrying antisense (or sense) versions of the rat IRS-1 cDNA (78%) or a synthetic 57-mer targeted at the initiation site. This vector is equipped to produce high-level constitutive transcription from mammalian enhancer/promoter sequences (CMV). In addition to other features not mentioned, it contains the neomycin resistance gene for selection of G418 resistant stable eukaryotic clones. The orientation of the insert in these constructs has been verified by restriction enzyme digestion and automated sequencing of the constructs is in progress. Sense orientation, as well as transfection performed with the vector alone, have served as negative controls. pcDNA3 and its precursor vector, pcDNA1/neo, have been successfully employed for the selection of stable transfectants by several laboratories. In addition, successful episome-based antisense strategies have been reported against IRS-1 to ablate the insulin-induced mitogenic response in a stable CHO cell line, as well as against IGF-1, which resulted in a loss of tumorigenicity of rat glioblastoma. This strategy, therefore, has not been designed without precedence.

To date, cationic liposome-mediated transfection (with Lipofectamine, Gibco, BRL) has been completed in the F442A cell line, and selection and propagation of single clones stably expressing the constructs has been achieved. Analysis by SDS-PAGE, Western blot, and densitometry has revealed that the antisense constructs exhibit a 52% (cDNA) and 27% (57-mer) reduction in IRS-1 protein content relative to the mock transfection control. Our antisense constructs seem, therefore, to be effective in achieving the desired goal of a significant decrease in IRS-1 protein levels.

These preliminary studies are being extended to several breast cancer cell lines. Four of the initial eight breast cancer cell lines screened are being retained for further studies based upon basal IRS-1 content (W. blot) and estrogen receptor (ER) status, an important indicator of the success of breast cancer therapy. The cell lines to be examined are: (1) BT20 (low IRS-1, ER+ but non-functional); (2) SKBR3 (moderate IRS-1, ER to be determined); (3) T47D (moderate IRS-1, ER+); and (4) MDA-MD-231 (high IRS-1, ER-). Since the candidate will be focusing on mitogenic assays, these cell lines are being grown in RPMI media (+10% FBS, +1% penicillin/streptomycin) minus phenol red, which has been shown to exhibit estrogen-like effects on estrogen-sensitive cell lines. Based on the preliminary Western blots performed to determine IRS-1 content, an apparent shift in mobility of IRS-1 was noticed in these breast cancer cell lines compared to the typical mobility of the basal state. Similar mobility shifts have been observed with insulin treatment in other systems and are consistent with tyrosine-phosphorylated IRS-1. We

are currently investigating if IRS-1 is tyrosine phosphorylated (and therefore activated) in the basal state in these cell lines, and if so, does this contribute to their tumorigenic potential?

Other experiments, designed to examine the mitogenic effect of insulin or IGF-1 acting through IRS-1 in these cell lines, are of relevance. One such assay involves the activation of phospholipase D (PLD), which results in the liberation of diacylglycerol (DAG) and phosphatidic acid (PA). Both of these second messengers are well known for their mitogenic potential. If IRS-1 is critical in eliciting this mitogenic effect, then the cells containing the antisense constructs should exhibit less PLD activation. Initial experiments examining the activation of PLD are already underway in our laboratory.

Future Work:

Experiments to be performed in the second year of funding on this grant include transfection of the breast cancer cells with the aforementioned antisense and sense constructs. A more marked decrease may perhaps be achieved by utilizing a similar vector now available to us that contains a b-globin intron between the CMV promoter and the insert that has been shown to increase transcription off of this promoter. Another approach which we plan to test, recently reported in the literature, is the utilization of high dose insulin (1 mM) treatment for 24 hours, which results in a virtually specific 90% reduction in IRS-1 protein.

Of additional interest, a series of murine hematopoietic progenitor cell lines (32D) have recently been made available to us. The parental cell line does not contain any IRS-1 and low levels of IR, while the three variants have been transfected with IR, IRS-1, or both, respectively. These cells will allow us to specifically address the involvement of IRS-1 in our future work. Mitogenic assays that measure the activation of PLD, PI3K and MAPK will also be performed in these cells, and results obtained herein should help direct us in our experiments with the breast cancer cell lines.

Other endpoints to be monitored in our transfected cells will be cellular changes in morphological appearance, growth rate, serum dependence, growth in semi-solid medium (0.41% agar) and eventually, experiments in nude mice to assess the tumorigenic potential of the transfected cells containing the antisense constructs. It is envisioned that a decrease in the transformed phenotype will be realized via this strategy. The mitogenic effect of activating the insulin signal transduction pathway plays a pivotal role in the pathobiology of certain breast cancers, and ultimately, IRS-1 may be targeted effectively for gene therapy of those breast cancers.

APPENDIX

Manuscript P95/039 Revised

Identification of a 2-D Geometric Descriptor Associated with Non-Genotoxic Carcinogens and Some Estrogens and Antiestrogens

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Summary

A distance descriptor (6Å) originally associated with non-genotoxic mouse carcinogens has been found to be present in some, but not all, estrogens and antiestrogens. It is hypothesized that this descriptor describes a ligand binding site on an estrogen receptor. Evidence is presented that those estrogens and antiestrogens not containing the 6Å distance bind to a different receptor. It is conceivable that binding to the receptor that recognizes the 6Å distance is associated with carcinogenicity.

Introduction

The extension of SAR methods to the elucidation of the action of non-genotoxic carcinogens is controversial. Thus, based upon the electrophilic theory of cancer causation (Miller and Miller, 1977) there is agreement that SAR approaches are useful for studying genotoxic carcinogens. There are, however, differences as to the appropriateness of applying SAR methods to the study of non-genotoxic carcinogens. The perceived obstacle to the successful use of SAR derives from the recognition that there is no single mechanism responsible for the action of non-genotoxic carcinogens (IARC, 1992; Ashby, 1992, 1994; Rosenkranz, 1992). In the present study we identify a geometrical descriptor that appears associated with non-genotoxic carcinogens and which, in fact, may represent a ligand binding site on an estrogen receptor. Moreover, "genotoxic" carcinogens which are defined operationally as rodent carcinogens that are *Salmonella* mutagens and/or possess "structural alerts" for DNA reactivity (Ashby and Tennant, 1991) are generally assumed to pose a greater risk to humans than "non-genotoxic" ones (Ashby and Morrod, 1991). Indeed, the

great majority of recognized human carcinogens are "genotoxic" (Ennever *et al.*, 1987; Shelby, 1988; Bartsch and Malaveille, 1989). The major exception to this generalization are the hormonal carcinogens (*e.g.*, diethylstilbestrol, estradiol) which are thought to act by receptor-mediated mechanisms (Barrett, 1992; Lucier, 1992; IARC, 1992). However, the recent reports that diethylstilbestrol (Gladek and Liehr, 1989; Williams *et al.*, 1993) and tamoxifen (Montandon and Williams, 1994; Han and Liehr, 1992; White *et al.*, 1992; Hard *et al.*, 1993) form DNA-adducts have generated renewed interest in the basis of the carcinogenicity of this group of agents. Recent studies in our laboratories have been concerned with the structural basis of the carcinogenicity of "genotoxic" and "non-genotoxic" carcinogens and their metabolites using the expert systems CASE/MULTICASE and META (Rosenkranz and Klopman, 1995). Indeed, in a recent study of the carcinogenicity of DES and its known and putative metabolites, we were unable to identify either mutagenic or electrophilic metabolites that could be the basis of a "genotoxic" mechanism of carcinogenicity (Cunningham and Rosenkranz, 1994). In the course of these and related studies, we did, however, obtain evidence that the carcinogenicity of DES in mice was associated with a lipophilic 6Å geometric distance descriptor (Cunningham and Rosenkranz, 1995). Since this could reflect the presence of a ligand binding site, we further investigated the nature of the chemicals which contain this descriptor. The results of that study are reported herein.

Material

Expert System: CASE/MULTICASE

For the present investigation, we used the MULTICASE (MC) program (Klopman, 1992; Klopman and Rosenkranz, 1994). Basically, MC selects its own descriptors automatically from a learning set composed of active and inactive molecules. The descriptors are readily recognizable single, continuous structural fragments that are embedded in the complete molecule. The descriptors consist of either activating (biophore) or inactivating (biophobe) fragments. Each of these fragments is associated with a confidence level and a probability of activity which is derived from the distribution of these biophores and biophobes among active and inactive molecules.

Upon completion of these analyses, MC selects the most important of these fragments as a biophore, *i.e.*, the functionality that is responsible for the experimentally observed activity of the molecules that contain it. MC then, using the molecules containing this biophore, will use them as a learning set to identify the chemical properties (*i.e.*, structural fragments) or physical chemical properties (*e.g.*, log P, water solubility, quantum mechanical parameters such as HOMO and LUMO, etc.) that modulate (either augment or decrease) the activity of the initially identified biophore. This will result in a QSAR equation for this subset of molecules. If the data set is congeneric, then the single biophore and associated modulators may explain the activity of the entire training set. This will usually not occur and there will be a residue of molecules not explained by the single biophore and related modulators. When this happens, the program will remove from consideration the molecules already explained by the previous biophore and will search for the next biophore and

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associated modulators. The process is continued until the activity of all of the molecules of the learning set has been explained.

The resulting list of biophores is then used to predict the activity of yet untested molecules. Thus, upon submission for evaluation, MC will determine if an unknown molecule contains a biophore. If it does not, the molecule will be predicted to be inactive unless it contains a group that chemically resembles one of the biophores, in which case it will be flagged. When the molecule contains a biophore, the presence of modulators for that biophore will be investigated. MC will then make qualitative as well as quantitative predictions of the activity of the unknown molecule.

Obviously, while biophores are the determining structures, the modulators may determine whether and to what extent the biological potential of the chemical is expressed.

Additionally, MC incorporates the following rules to identify two-dimensional distance descriptors based upon the presence of lipophilic centers. These two dimensional distances are calculated from the molecular structure. Heteroatoms and lipophilic carbon atoms are designated as "special" atoms. A carbon atom is designated as a lipophilic center if it is at least four bonds away from a heteroatom and is also the furthest carbon away from the heteroatom when its neighbors are considered. After all the "special" atoms are identified, the distances between all possible pairs are calculated.

The distribution of these descriptors among active and inactive molecules is analyzed for statistical significance. If the atoms at both ends of the distance descriptor are all the same, including the number of attached hydrogens, the biophore is designated an "exact"

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descriptor. Various atom groupings are also investigated, *i.e.*, hydrogen bond acceptors and donors as well as halogens.

Expert System: META

The expert system "META", a computer based metabolism program, was used to investigate the metabolism of tamoxifen and tomerifene. The META program has been recently described in detail (Klopman *et al.*, 1994; Talafous *et al.*, 1994). Briefly, META contains a knowledge set of 665 enzyme-catalyzed and 286 spontaneous reactions which constitute most of the phase I and II metabolic pathways. When presented with the structure of a parent molecule, META indicates a series of possible metabolic pathways which include a graphical description of each possible metabolite(s) and intermediate metabolite(s) as well as the enzymes used to generate these.

Mouse carcinogenic potency database

The carcinogenic potency database was assembled by Gold *et al.* (1984, 1986, 1987, 1990, 1993). A subset of mouse carcinogens (males and females) was derived from this compilation and subjected to MC analysis. In that database, chemicals reported as carcinogenic by the primary authors are accompanied by their TD_{50} values, *i.e.*, the dose required for 50% of the animals to remain cancer free (Gold *et al.*, 1984; Peto *et al.*, 1984). These were transformed into gavage equivalents(Gold *et al.*, 1984, 1986; Brown and Ashby 1990). Additionally, the TD_{50} values (in mg/kg/day) were converted in mmol/kg/day. Using Equation 1 (see below), chemicals were assigned to activity groups.

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The database consists of 639 chemicals, 291 of which are carcinogens, 11 are marginal and 337 are non-carcinogens. Chemicals reported by the authors to be non-carcinogenic in mice were assigned 10 CASE units together with chemicals with a TD_{50} value in excess of 51 mmol/kg/day.

For the purpose of the SAR analysis, TD_{50} values (*i.e.*, potencies) in mmol/kg/day were transformed into CASE units using the following relationship:

$$\text{CASE activity} = 14.1329 * (\log 1) / (TD_{50} + 44.1329) \qquad \text{(Equation 1)}$$

In order to accomodate the broad range of TD values present in the data base, we chose to relate the TD_{50} values to CASE units in a logarithmic function. Thus, using Equation 1, chemicals in the range of 10 to 19 CASE units are inactive or exhibit negligible activity. Chemicals with activities in the range of 20 to 29 CASE units are seen as marginally active and chemicals in the range of 30 to 99 CASE units are called carcinogenic.

It should be noted that since the data base is for carcinogenicity in mice (males and females), the MULTICASE predictions do not specify the gender involved. Moreover, in the analyses no attempt was made to predict tissue-specificity.

Results and Discussion

The 6Å descriptor (see Figure 1) was originally identified as a biophore associated with carcinogenicity in mice (Cunningham and Rosenkranz, 1995). That finding was based upon the Carcinogen Potency Database of Gold *et al.* (1984, 1986, 1987, 1990, 1993). Among the chemicals in the data base which contain this descriptor was estradiol and related

chemicals (Figure 1), thus suggesting that the 6Å descriptor may be associated with estrogenicity. In order to determine whether this was the case, we tested a series of chemicals reported to be endowed with estrogenicity for the presence of the 6Å descriptor. A number, but not all, of them displayed this property, suggesting that, indeed, this descriptor is related to estrogenicity. It should be noted that while the therapeutically useful anticancer agents tamoxifen and toremifene lack the 6Å descriptor (Table 1), some of their metabolites contain it. Indeed, among these are the metabolites which are believed to be responsible for the estrogenicity of tamoxifen (see Lerner and Jordan, 1990). If it be assumed that the 6Å descriptor identifies not only a biophore associated with murine carcinogenicity, but also an estrogen-receptor ligand, then the presence of this moiety in tamoxifen metabolites may explain the carcinogenicity as well as the estrogenicity of the parent molecule. Moreover, this suggests that the two phenomena may result from the same mechanism, *i.e.,* binding to a specific site. If this is so, then it could be advantageous to design antiestrogens which lack the 6Å descriptor and therefore bind to another receptor. In order to determine whether this is a viable alternative, we tested a number of estrogens and antiestrogens. It is of interest to note that some of these (*e.g.,* ICI 164,384 and ICI 182,780) contain the 6Å descriptor (Figure 2) while others (*e.g.,* LY 117018) do not (see Table 1). It has already been suggested that LY117018 and tamoxifen (Black and Goode, 1981; Scholl *et al.* 1983) and LY 117018 and ICI 164,384 / ICI 182,780 (Coradini *et al.* 1994) have different bases for their antiestrogenicity. The present findings together with the previously reported dichotomy of the action of antiestrogens, suggests that the latter may act by different mechanisms. Our findings further suggest the possibility that the 6Å distance

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descriptor identifies a ligand binding site on one of the estrogen receptors. If indeed the 6Å biophore is associated with rodent carcinogenicity, then perhaps targeting the estrogen receptor which does not recognize this moiety may be a promising endeavor for the development of therapeutically useful antiestrogens.

As mentioned earlier, the 6Å biophore was first identified using a rodent carcinogenicity data base which is not congeneric with respect to chemical species. Accordingly, the modulators identified as associated with this biophore may not provide a complete understanding of the factors affecting activity.

In order to further explore the nature of the 6Å biophore, the data base was supplemented with a series of diverse molecules containing these descriptors (N = 31) as well as with congeners lacking it (N = 9). Upon analysis of this "synthetic" learning set with MULTICASE, the 6Å biophore was once again identified ($p < 0.01$). However, the nature of the modulators associated with this biophore permitted a further delineation of its nature. Thus, all of the modulators are within the 6Å domain and describe moieties that either enhance or decrease the projected activity (see Figure 3). Moreover, the physical chemical modulator affecting this biophore is the log P (octanol:water partition coefficient) which further substantiates that the 6Å biophore encompasses a lipophilic center. Thus, the modulators are consistent with the possibility that the 6Å biophore describes a ligand which is recognized by a specific receptor.

Finally, in order to gain an understanding of the possible distribution of the 6Å biophore among molecules, we determined its presence among molecules (N = 5400) representing the "universe of chemicals". Some of the chemicals containing this moiety are

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listed in Table 2 and it is of interest that it is present in a wide selection of agents, some of which are in widespread use (*e.g.*, BHT), others which are used in cancer chemotherapy (*e.g.*, NSC377163) and still others which are used in traditional medicine (*e.g.*, hemigossypol). Some of these might be endowed with estrogenicity. It is of further interest that some hydroxylated metabolites of polycyclic aromatic hydrocarbons (*e.g.*, 11-hydroxypyrene, 5-hydroxydibenz (a,h)anthracene, 3-hydroxy-7,12-dimethylbenz(a)anthracene) contain the 6Å biophore (Table 2). The estrogenicity of polycyclic aromatic hydrocarbons is a recognized phenomenon (Davis *et al.*, 1993; Safe, 1995).

The current findings suggest a number of additional studies that should lead to a further refinement of the biophore as well as a better understanding of its relevance to the induction of cancer and estrogenicity. Moreover, the recognition of at least two classes of estrogens differing in a geometric descriptor may enable us to identify the nature of the ligand which binds to the other estrogen receptors.

The present report documents the existence of a structural descriptor which appears to be related to non-genotoxic carcinogens. Moreover, the new biophore implies a mechanism of action as well. This indicates the feasibility of applying SAR approaches to non-genotoxic carcinogens.

Acknowledgments

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TABLE 1

Distribution of 6Å Descriptors Among Estrogens, Xenoestrogens, and Antiestrogens

CHEMICAL	Type	6Å Descriptor
o,p'-DDE	Xenoestrogen	-
1-Hydroxy-E-X-diethylstilbestrol	Estrogen metabolite	+
17-α-Ethinyl estradiol	Estrogen	+
2'-Hydroxygenistein	Phytoestrogen	-
3'-Hydroxy-E-diethylstilbestrol	Estrogen metabolite	+
3'-Methoxy-E-diethylstilbestrol	Estrogen metabolite	+
3-Hydroxytamoxifen	Antiestrogen metabolite	-
4',4"-Diethylstilbestrol quinone	Estrogen metabolite	-
4-Hydroxytamoxifen acid	Antiestrogen metabolite	+
4-Hydroxy-deamino-hydroxytoremifene	Antiestrogen metabolite	+
4-Hydroxytoremifene	Antiestrogen metabolite	+
Benzestrol	Estrogen	+
Chlordecone	Xenoestrogen	-
Coumestrol	Phytoestrogen	-
Dienestrol	Estrogen	+
Diethylstilbestrol	Estrogen	+
Estriol	Estrogen	+
Estrone	Estrogen	+
Genistein	Phytoestrogen	-
Hexestrol	Estrogen	+
Indenestrol A	Phytoestrogen	+
Megestrol	Estrogen	-
Mestranol	Estrogen	-
Norgestrel	Estrogen	-
Norlestrin (Isomer A)	Estrogen	+
Phenol red	Estrogen	-
Tamoxifen	Antiestrogen	-
Tamoxifen-bis-phenol	Antiestrogen metabolite	+
Tetrahydrocannabinol	Xenoestrogen	-
Toremifene	Antiestrogen	-
Zearalenone	Xenoestrogen	-
β-Estradiol	Estrogen	+
z-Bisdehydrodoisynolic acid	Phytoestrogen	+
Allenolic acid	Phytoestrogen	+
Kaempferol	Phytoestrogen	-
Quercetin	Phytoestrogen	-

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TABLE 1 continued

Distribution of 6Å Descriptors Among Estrogens, Xenoestrogens, and Antiestrogens

Antiestrogens	Type	6Å Descriptor
ICI 164,384	Antiestrogen	+
ICI 182,780	Antiestrogen	+
LY 117018	Antiestrogen	-
MER 25 (Ethamoxytriphetol)	Antiestrogen	-
3-Phenylacetylamino-2,6-piperidinedione	Antiestrogen	-
p-Hydroxy-3-phenylacetylamino-2,6-piperidinedione	Antiestrogen	-

Additional hydroxylated putative metabolites of tamoxifen and toremifene also contain the 6Å structural descriptor.

TABLE 2
Some Chemicals Containing the 6Å Descriptor

11-Hydroxybenzo(a)pyrene
1,3,4-Xylenol
2-Amino-4-methylphenol
2-Amino-4-methylphenol
2,3-Dihydroxynaphthalene
2,4-Dimethylphenol
2,4,6-Trimethylphenol
3-t-Butyl-5-methylcatechol
3-t-Octyl-5-methylcatechol
3,4,5-Trimethylphenol
3,9-Dihydroxybenzanthrone
4-Hydroxy-desmethyl-toremifene
4-Methyl-2-nitrophenol
4-Methylcatechol
4-Methylphenol (p-Cresol)
4,4'Dihydroxy-3-(hydroxymethyl) diphenylmethane
4,5-Dimethyl-3-pentadecylcatechol
4,5-Dimethylcatechol
5-Hydroxyacenaphthene
7-Hydroxy-4-isopropyltropolone
Allylsyringol
Aucuparin
Butylated Hydroxytoluene (BHT)
C.I. Disperse Yellow 3
C.I. Pigment Red 3
Calmagite
CI Pigment Red No. 23
CI. Pigment Red No. 23
Citrus Red 2
D and C Red No. 10
D and C Red 9
Desoxyhemigossypol
Diflunisal
Eugenol
Fast Green FCF
Gamma-thajaplicin
Gossypol
Hemigossypol
Levallorphan

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TABLE 2 (continued)
List of Chemicals Containing the 6Å Descriptor

NSC 377163 (Pyrazoloacridine)
Oxymetazoline
p-Phenylphenol
Purpurogallin
Scarlet Red
Violaceol-1
Viridicatumtoxin

The molecules listed above were selected from among a group of 5,400 molecules representative of the "universe of chemicals" because they contain the 6Å descriptor. In this analysis no consideration was given to the possible metabolism of these or the other molecules in the testing set. The 5,400 chemicals tested represent a random selection of chemicals taken to reflect the "universe of chemicals" (National Academy of Sciences, 1984)

The molecule contains the Biophore (number of. occurrences = 1):

biophore A: 2D fragment: [C -] <-- 6.0A --> [OH -] conj generic

   14 out of the known 16 molecules (87%) containing such a Biophore are Mouse carcinogens with an
   average activity of 47 CASE unit (conf.level=100%).

                                                            Constant is 51.8

The following Modulator is also present:

modulator 1:   OH - CH -   Activating 34.2

   The probability that this molecule is a Mouse carcinogen is 83.3% -

   The compound is predicted to be EXTREMELY active
   The projected Mouse carcin activity is 86.0 CASE units
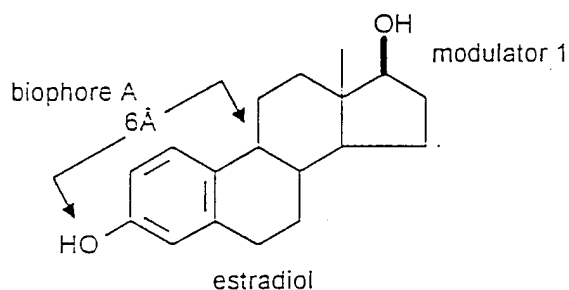
Figure 1. Prediction of the carcinogenicity in mice of estradiol. The biophore and modulator are indicated.
A potency of 86 CASE units indicates a TD_{50} value of 0.001 mmoles/kg/day.

The molecule contains the Biophore (number of. occurrences = 1):

biophore A: 2D fragment: [C -] <-- 6.0A --> [OH -] conj generic

    14 out of the known 16 molecules (87%) containing such Biophore are Mouse carcinogens with an average activity of 47 CASE units (conf.level=100%).

                                                               Constant is 51.8

The following Modulator is also present:

modulator 1:   OH - CH -   Activating 34.2

The molecule also contains the Biophore:

biophore B: CH2-CH2-CH2-CH -

    The probability that this molecule is a Mouse carcinogen is 83.3% increased to 89.8% due to the presence of the extra Biophore

    The compound is predicted to be EXTREMELY active
    The projected Mouse carcin activity is 86.0 CASE units
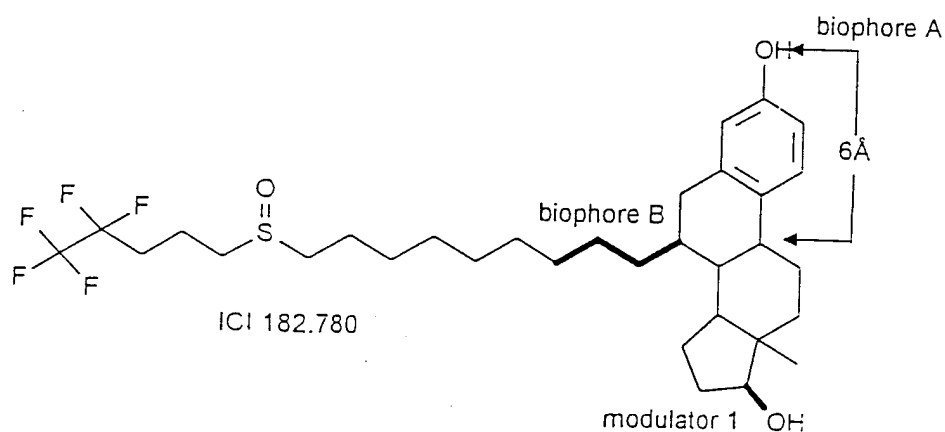
Figure 2. Prediction of the carcinogenicity in mice of the antiestrogen ICI 182,780. The two structural descriptors are indicated. A potency of 86 CASE units can be translated into a TD50 of 0.001 mmoles/kg/day.

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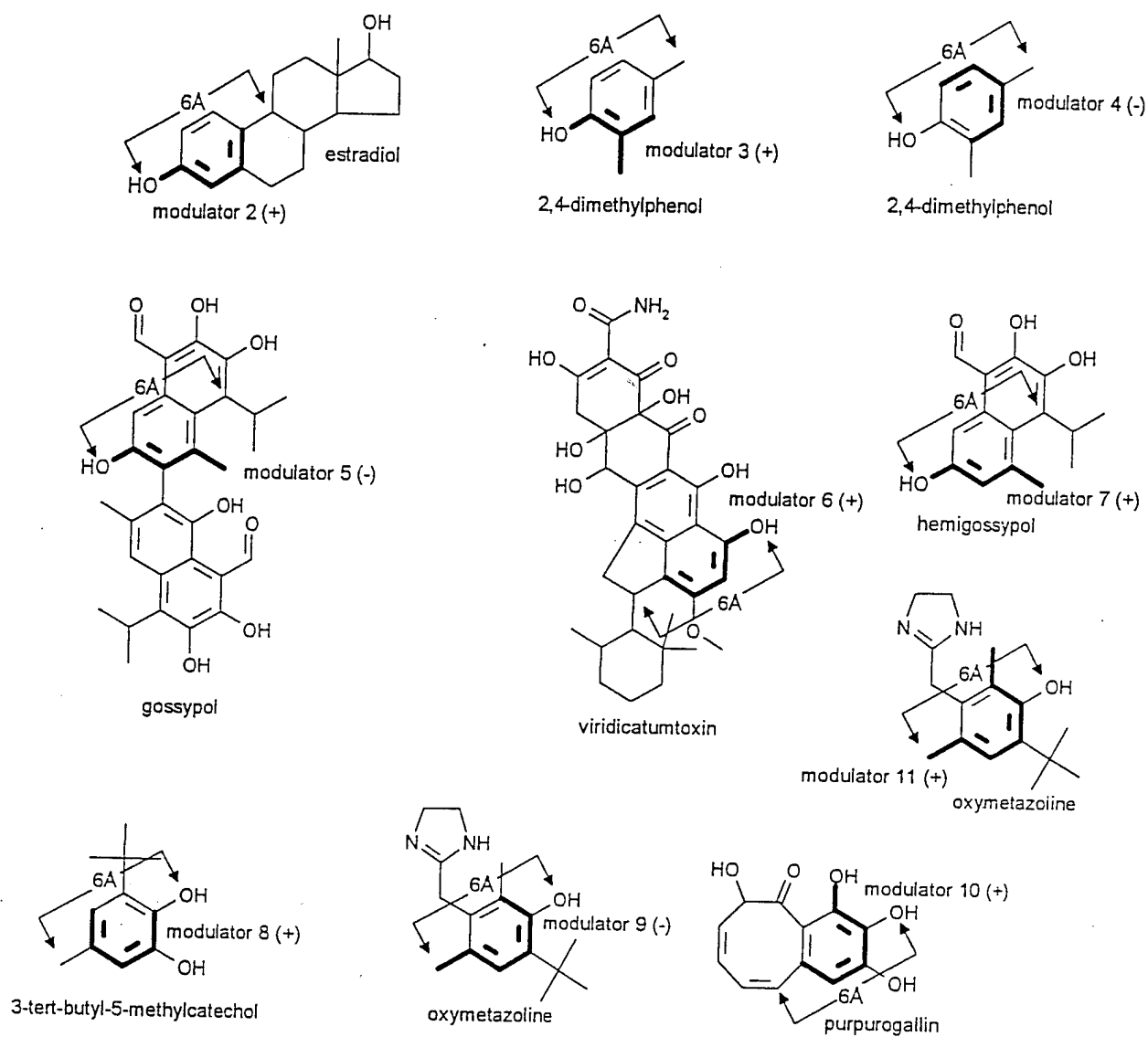
Figure 3. Description of the modulators associated with the 6Å biophore. (+) and (-) next to the modulator designation indicates an activating or an inactivating modulator, respectively.

A STUDY OF THE STRUCTURAL BASIS OF THE CARCINOGENICITY OF TAMOXIFEN, TOREMIFENE AND THEIR METABOLITES

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Summary

An analysis of the chemical structure of tamoxifen, toremifene and their metabolites indicates that metabolism to a DNA-reactive hydroxylamine intermediate is possible. The parent compounds and many of their metabolites are predicted to be rodent carcinogens. Moreover, many of these metabolites contain a 6Å or 8.4Å distance descriptor biophore. These geometric descriptors may be related to an ability of these chemicals to bind to an estrogen receptor. The prediction of the carcinogenicity of toremifene is not in accord with studies published thus far. However, the reports available have not excluded this possibility since the protocols used have not addressed it systematically.

Introduction

Estrogens and their antagonists, the antiestrogens, are the subjects of debate regarding aspects of cancer etiology and therapy as well as other endocrine-related health effects. The distinction between estrogen and antiestrogen is obscure. Several of the factors that determine the estrogenicity *vs* antiestrogenicity of a chemical are species- and tissue-specific (for a review on antiestrogens, see Lerner and Jordan, 1990).

Antiestrogens, such as tamoxifen (i.e., antiestrogenic in human breast tissue), are pivotal to chemotherapeutic strategies for breast cancer. Newer antiestrogens are being introduced into breast cancer chemotherapeutic regimens such as the tamoxifen congener toremifene and the "pure" antiestrogens ICI 164384 and ICI 182780. Due to a decreased risk of developing contralateral breast cancer in patients receiving tamoxifen (Early Breast Cancer Trialists' Collaborative Group, 1992a, b; Fisher *et al.*, 1989), tamoxifen is presently undergoing trials as

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a prophylactic agent against breast cancer by the National Surgical Adjuvant Breast and Bowel Project (NSABP).

While antiestrogens are useful in the treatment and possible prevention of breast cancer, recently environmentally estrogens have been implicated in the etiology of breast cancer (Davis *et al.*, 1993). Phenols and halogenated hydrocarbons represent two groups of organic chemicals widely used as solvents and industrial intermediates. Human exposures to these chemicals can occur occupationally or environmentally. The toxicological properties of these chemicals include estrogenic and antiestrogenic activities and, accordingly, they may be classified as "xenoestrogens". Xenoestrogen exposure is implicated in certain reproductive anomalies in both humans and wildlife (Guillett *et al.*, 1994; Santti *et al.*, 1994; Sharpe and Skakkebaek, 1993). Moreover, if these agents act through a receptor that also recognizes physiological and therapeutic estrogens or their antagonists, it can be expected that they will exhibit at least an additive effect with respect to the activities of the former.

The purpose of the present investigation was to gain insight, through the structure-activity relationships (SAR), of the carcinogenic and therapeutic mechanisms of estrogens and antiestrogens with emphasis on the antiestrogens tamoxifen (TMX) and toremifene (TRM) (Figure 1). The present study is part of our continuing investigations of the bases of action of "non-genotoxic" carcinogens (Rosenkranz and Klopman, 1990a, 1993; McCoy *et al.*, 1990; Lee *et al.*, 1995).

TMX and TRM are relevant to human carcinogenesis due to their similarities with diethylstilbestrol (reviewed by IARC, 1987; Marselos and Tomatis, 1992). TMX has been

implicated in the induction of rare endometrial cancer in humans (Cohen et al., 1993; Fornander et al., 1989; Gal et al., 1991; Gusberg, 1990). In experimental animals TMX induces hepatocellular carcinomas and hepatic adenomas (Hard et al., 1993a,b; Hirsimaki et al., 1993; Greaves, et al., 1993; Williams et al., 1993). It has been suggested that TMX acts as a "genotoxic" carcinogen due to its ability to form DNA adducts as detected by the ^{32}P-postlabeling technique (Hard et al., 1993a; Han and Liehr, 1992, Montandon and Williams, 1994; White et al., 1992; Phillips et al., 1994) and the induction of micronuclei in cultured cells (Styles et al., 1994; White et al., 1992). These findings have direct relevance to human risk, as "genotoxic" carcinogens are thought to present a greater risk than "non-genotoxic" ones (Ashby and Morrod, 1991). In fact, the vast majority of recognized human carcinogens are genotoxicants (Ennever et al., 1987; Bartsch and Malaveille, 1989; Shelby, 1988).

The mechanism of TMX reactivity with DNA has not been elucidated. Styles et al. (1994) have reported that TMX may be metabolized to DNA-reactive epoxides. However, α-hydroxylation of the ethyl group yielding an alkylating metabolite has been postulated (Potter et al., 1994) and experimental evidence has been presented in support of this mechanism (Phillips et al., 1994; Randerath et al., 1994a,b).

TRM is also endowed with estrogenic and antiestrogenic properties (Homesley et al., 1993; Stenbygaard et al., 1993, Valavaara et al., 1988; Vogel et al., 1993). Unlike TMX, TRM is reported to be 'non-genotoxic'(i.e., no adducts by ^{32}P-postlabeling technique) (Montandon and Williams, 1994) and to be non-carcinogenic to rodents under conditions in which TMX induces liver tumors in rats (Tucker et al., 1984). Thus comparison of TMX and TRM could permit a

determination of the mechanism of carcinogenicity of this group of chemicals, especially with respect to the possibility that their estrogenicity and carcinogenicity are separate phenomena.

Methods

CASE/MULTICASE methodology

The CASE (Klopman, 1984; Klopman and Rosenkranz, 1984) and MULTICASE (MC) (Klopman, 1992; Klopman and Rosenkranz, 1994) methodologies has been described on a number of occasions. Basically, MC selects its own descriptors automatically from a learning set composed of active and inactive molecules. The descriptors are readily recognizable single, continuous structural fragments that are embedded in the complete molecule. The descriptors consist of either activating (biophore) or inactivating (biophobe) fragments. Each of these fragments is associated with a confidence level and a probability of activity that is derived from the distribution of these biophores and biophobes among active and inactive molecules.

Additionally, MC incorporates the following rules to identify two-dimensional distance descriptors based upon the presence of lipophilic centers. These two-dimensional distances are calculated from the molecular structure. Heteroatoms and lipophilic carbon atoms are designated as "special" atoms. A carbon atom is designated as a lipophilic center if it is at least four bonds away from a heteroatom and is also the farthest carbon away from the heteroatom when its neighbors are considered. After all the "special" atoms are selected the distances between all possible pairs are calculated. The distribution of these descriptors among active and inactive molecules is analyzed for statistical significance. Various atom groupings are also investigated, *i.e.*, hydrogen bond acceptors and donors as well as halogens.

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Metabolites

A group of experimentally identified metabolites of TMX and TRM was assembled from the literature (Figure 1). In conjunction with this, the expert system "META", a computer based metabolism program, was employed (Klopman, *et al.*, 1994; Talafous, *et al.*, 1994). META contains a knowledge set of 655 enzyme-catalyzed and 286 spontaneous reactions that include most of the phase I and II enzymes. When META is presented with the structure of a parent molecule it indicates a series of possible metabolic pathways and a structural representation of the metabolites.

The parent compounds were "metabolized" through one iteration which yielded a group of metabolites. Each non-conjugated metabolite, was again "metabolized" and so on through four iterations. Overall, 132 TMX and 182 TRM non-conjugated experimental and putative metabolites were assembled. A description of these putative metabolites can be found elsewhere (Cunningham and Rosenkranz, 1995).

Databases

TMX, TRM and the set of metabolites were analyzed by MC using carcinogenicity and mutagenicity databases. The rules for inclusion of chemicals in the databases (*i.e.*, learning sets) have been described previously (Rosenkranz and Klopman, 1990a, c).

The *Salmonella* mutagenicity database (SMDB) was generated under the aegis of the U.S. NTP (Ashby and Tennant, 1991; Cater *et al.*, 1986; Haworth *et al.*, 1983, 1989; Lawlor, *et al.*, 1985; Mortelmans *et al.*, 1984, 1986; Reid *et al.*, 1984; Zeiger, 1990; Zeiger and Haworth, 1985; Zeiger *et al.*, 1985, 1987, 1988). The database consisted of 1354 chemicals. A SAR

analysis of a subset of these chemicals has been reported previously (Rosenkranz and Klopman, 1990b).

The rodent carcinogenicity database (CDB) was generated under the aegis of the U.S. NTP. For the purpose of the present analyses we used the summaries of the bioassays on 301 chemicals (Ashby and Tennant, 1991). Due to the ambiguous nature of chemicals classified as "equivocal carcinogens" and chemicals removed because of structural limitations, 255 chemicals were used for the analysis. The six levels of carcinogenicity (A-F) (Ashby and Tennant, 1991) have been converted to 10-60 CASE units, respectively. A SAR analysis of a subset of these chemicals has been reported previously (Rosenkranz and Klopman, 1990c).

The carcinogenic potency database (CPDB) was assembled by Gold *et al.* (1984, 1986, 1987, 1990, 1993). Three subsets were derived from this compilation (rodent, mouse and rat). In contrast to the CDB, this compilation is based primarily on published reports. Presumably these were not subjected to the same rigorous quality assurance and peer review tiers of the NTP. However this database has two advantages with respect to the present study:

   a. For chemicals judged to be carcinogenic, TD_{50}, the dose required for 50% of the animals to remain cancer free, was calculated (Gold *et al.*, 1984; Peto *et al.*, 1984).

   b. The number of chemicals included in this database is greater than that included in the CDB. This results in an increased informational content of the database as well as allowing refinements in the nature of the structural determinants.

For each database all dosages reported were transformed into gavage equivalents. Additionally, the TD_{50} value in mg/kg/day was converted into mmol/kg/day. Using the appropriate equations (see below) potencies were transformed into CASE units and the chemicals were assigned to activity groups.

The mouse carcinogen subset of the CPDB consisted of 639 chemicals, 291 of which are active, 11 are marginal and 337 are non-carcinogenic. The rat carcinogen subset of the CPDB consisted of 744 chemicals, 380 of which are active, 15 are marginal and 349 are non-carcinogenic.

The rodent carcinogen subset of the rodent CPDB consisted of 437 chemicals, 265 of which are active, 8 are marginally active and 164 are inactive. To be included in this database chemicals had to be tested both in the rat and the mouse. If the chemical was determined to be carcinogenic in both rats and mice the value for the more sensitive species, *i.e.*, the lower TD_{50} value, was used.

For the purpose of the SAR analyses TD_{50} values in mmol/kg/day were transformed into CASE units using the following relationships:

$$\text{CASE unit} = 14.1329 * (\log 1 / TD_{50}) + 44.1329 \quad \text{(mouse CPDB)}$$

$$\text{CASE unit} = 20.124 * (\log 1 / TD_{50}) + 44.066 \quad \text{(rat CPDB)}$$

$$\text{CASE unit} = 18.3279 * (\log 1 / TD_{50}) + 46.5517 \quad \text{(rodent CPDB)}$$

Chemicals in the range of 10 to 19 CASE units are inactive or exhibited negligible carcinogenicity. Chemicals with activities in the range of 20 to 29 CASE units are marginally active and chemicals in the range of 30 to 99 CASE units are carcinogenic.

Results

MC analysis using the SMDB indicates that TMX and TRM have a potential for mutagenicity. MC identified the moiety -CH_2-Cl present in TRM and many of its metabolites as an indication that TRM may be mutagenic (Table 1). Examination of the configuration of the -CH_2-Cl fragment among the 37 mutagenic chemicals containing it indicates that their configurations are greatly different from that of TRM. The mutagens that possess -CH_2-Cl are primarily haloalkanes, esterfied phosphates or nitrogen mustards, and act through a DNA alkylating mechanism. Additionally, the -CH_2-Cl moiety may not be always related to the mutagenicity of chemicals that contain it. The mutagenicity of these chemicals may be derived from other functionalities present in the molecule, *e.g.*, nitro functionalities, as in nitrobenzyl chloride and epoxy functionalities, as in 1,2-epoxy-3-chloropropane. In fact, the chemical in the database that most resembles the configuration of the -CH_2-Cl moiety of TRM is the *non-mutagenic* tetrachlorodiphenylethane. (For a list of the chemicals containing the -CH_2-Cl moiety, see Cunningham and Rosenkranz (1995)).

MC identified a hydroxylamine moiety, OH-NH- (Table 1). This biophore is present in several of the META derived metabolites from both TMX and TRM. Considering the presence of this fragment, MC predicted that these metabolites may be mutagenic. Aliphatic hydroxylamines are known mutagens capable of reaction with deoxycytidine (Freese, 1963) while

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arylhydroxylamines form adducts with the O-6 (Kadlubar et al., 1978) or C-8 (Beland et al., 1983) of deoxyguanosine. Several metabolic pathways were identified by META which produce hydroxylamine metabolites. Details of the metabolic transformations can be found in Cunningham and Rosenkranz (1995). The putative metabolites that contain this biophore are related to desmethyl TMX and desmethyl TRM (Figure 1). They can be considered aliphatic hydroxylamine analogs. It is noteworthy that a recent report of TMX-induced DNA adducts has identified deoxyguanosine as the primary target (Martin et al., 1995).

Our analyses indicate a pathway for the generation of potential DNA reactive metabolites for TMX and TRM. Whether these explain the adducts detected by the ^{32}P-postlabeling technique is problematic, only TMX and not TRM are reported to give rise to such adducts. Moreover, in the case of DES treatment that yield adducts, it has been shown that they reflect endogenous chemicals which may result from hormonal stress (Liehr et al., 1986).

MC analysis of TMX, TRM and their metabolites identified a series of structural features indicative of potential carcinogenicity in both the CDB and the rodent CPDB (Table 1). The parent compounds, as well as many of the metabolites, posses the biophore CH=C-C=C- identified by the CDB and the closely related biophore, CH=CH-C=C-C= identified by the rodent CPDB (see Table 1). Analyses based on the CDB identified in some metabolites the biophores C=CH-C=C and CH-CH-C(-O)=C-CH=. Lastly, the rodent CPDB-based analyses indicated the $-CH_2-Cl$ moiety of TRM is predictive of carcinogenicity. However, the $-CH_2-Cl$ moiety can be dismissed as related to carcinogenicity for the same reasons it was eliminated as a descriptor for mutagenicity in Salmonella.

The projected potency of TMX and TRM based on the rodent CPDB is 48 and 46 CASE units, respectively. These values translate into TD_{50} values of 0.8 and 1.1 mmol/kg/day, which is in the same range as aflatoxin B1 and N,N-dimethylaniline. The projected potency based upon the CDB of TMX and TRM is 49 and 51 CASE units respectively. These values suggest that the parent molecules, TMX and TRM, are carcinogenic to a single species but at multiple sites. Additionally, based upon rodent CPDB, the range of predicted potencies of the metabolites is greater than for the parent molecule (Table 1). The range of potencies in the CDB respectively for TMX and TRM metabolites was 41-57 and 42-57 CASE units. These values are derived from two separate biophores. This suggests (see Cunningham and Rosenkranz, 1991) that some of the metabolites may have the potential to be carcinogenic in male and female rats and mice at multiple sites. Moreover, both the increased potencies and extended carcinogenic range suggests that the predicted carcinogenicity of TMX and TRM is due to their metabolites. It should be noted that these predictions are not completely in accord with published reports that indicate that TMX but not TRM is a rat carcinogen. However, TMX is a suspected human carcinogen based on its ability to cause cancer in several animal species. We could not find published reports of TMX and TRM cancer bioassays using an NTP-type protocol in which carcinogenicity to multiple species of both genders and examination of all tissues was included. Hence our predictions of the carcinogenicity of TRM remain to be verified

MC analysis of TMX and TRM in the mouse and rat CPDB found no evidence to predict that the parent molecules are carcinogenic. However, identified as well as putative metabolites of TMX and TRM possess biophores indicative of a potential for carcinogenicity (Table 1). Two of these are distance descriptors of 6.0Å and 8.4Å between a lipophilic carbon and a hydroxyl moiety associated with the mouse CPDB and the rat CPDB respectively. The structural

fragment =C (-OCH$_3$)-CH=C=C

present in one TMX metabolite is a carcinogenic biophore based upon the rat CPDB.

The putative metabolites of TMX and TRM predicted to be carcinogenic are so by virtue of two-dimensional distance descriptors. The possible significance of these distance descriptors may be related to the physical requirements of a ligand binding site on estrogen receptors. Further investigations are currently underway to better understand these descriptors since receptor-based mechanisms are thought to be important in the etiology of estrogen-induced cancer.

The projected potency of metabolites that contain the 6.0Å descriptor in the mouse CPDB is 52 CASE units for both TMX and TRM metabolites. This value translates into a TD$_{50}$ value of 0.28 mmol/kg/day. The projected potency of metabolites that contain the 8.4Å descriptor in the rat CPDB is 85 CASE units for both TMX and TRM metabolites that translates into approximately 0.01 mmol/kg/day.

Discussion

While TMX, TRM and the majority of their metabolites are predicted to be non-mutagenic in *Salmonella*, the possibility exists that metabolism of the putative desmethyl intermediates may yield hydroxylamines capable of modifying the deoxycytidine and deoxyguanosine moieties in DNA. Thus TMX and TRM are potentially genotoxic. This is supported by the finding that the major DNA-adduct in TMX-treated rats involves deoxyguanosine (Martin *et al.*, 1995).

In the two rodent databases TMX, TRM and their metabolites are predicted to be carcinogenic with an indication that metabolism is required to achieve the full carcinogenic potential, *i.e.*, metabolites of both have higher projected potencies and broader spectra of activity than the parent compounds.

Both TMX and TRM gain carcinogenic attributes associated with the mouse and rat CPDB. These observations suggest that metabolism is likely an important step in the carcinogenic potentiation of these two chemicals. In fact, 4-hydroxy-TMX has a higher binding affinity with the estrogen receptor than does TMX (reviewed by Lerner and Jordan 1990). For both the rat and mouse CPDB, hydroxylation at the *para* position is a requirement for the predicted carcinogenicity.

Although we found evidence that TMX has the potential for metabolism to a mutagenic hydroxylamine the preponderance of evidence from this investigation suggests that both TMX and TRM both may be carcinogenic by an alternate mechanism. *p*-Hydroxy metabolites of both TMX and TRM are predicted to be carcinogenic in the rat and mouse CPDB. In both instances this is due to distance descriptors. These biophores may be indicative of a receptor-based mechanism of estrogen-induced carcinogenesis.

Our present analyses suggest that TRM is a rodent carcinogen. This is not in accord with results reported heretofore. It should be noted, however, that the carcinogenicity of this antiestrogen has not been investigated systematically to rule out this possibility.

Acknowledgments

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tamoxifen

general form

toremifene
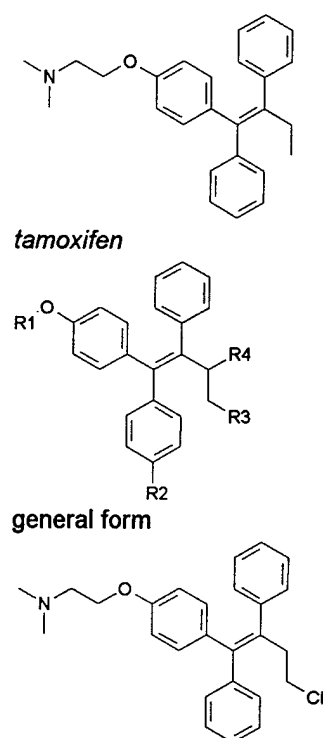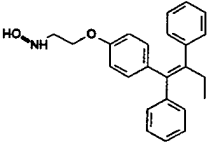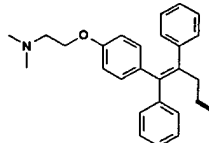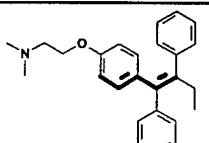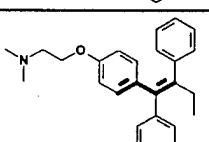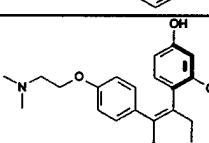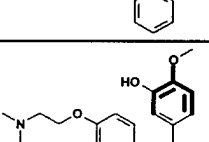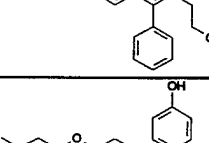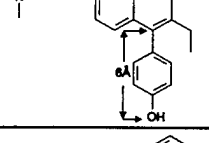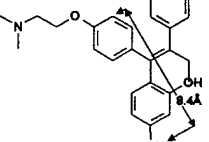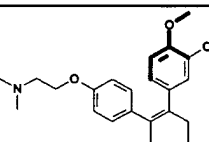
Name	R1	R2	R3	R4
tamoxifen (TMX)	$CH_2CH_2N(CH_3)_2$	H	H	H
N-desMe-TMX[1,2,3]	$CH_2CH_2NH(CH_3)$	H	H	H
N-didesMe-TMX[1,2]	$CH_2CH_2NH_2$	H	H	H
deamino-OH-TMX[1,2]	CH_2CH_2OH	H	H	H
4-OH-TMX[1,2,3]	$CH_2CH_2N(CH_3)_2$	OH	H	H
4-OH-N-desMe-TMX[1]	$CH_2CH_2NH(CH_3)$	OH	H	H
4-OH-deamino-OH-TMX[1]	CH_2CH_2OH	OH	H	H
TMX-N-oxide[1]	$CH_2CH_2NO(CH_3)_2$	H	H	H
α-OH-TMX-N-oxide[1]	$CH_2CH_2NO(CH_3)_2$	H	H	OH
α-OH-TMX[5]	$CH_2CH_2NH(CH_3)$	H	H	OH
TMX-bis-phenol[4]	H	OH	H	H
TMX acid[4]	CH_2COOH	H	H	H
TMX-glycine-OH[4]	CH_2CONCH_2COOH	H	H	H
4-OH-TMX acid[4]	CH_2COOH	OH	H	H
Me-4-OH-TMX-acid[4]	CH_2COOMe	OH	H	H
toremifene (TRM)[1]	$CH_2CH_2N(CH_3)_2$	H	Cl	H
N-desMe-TRM[1]	$CH_2CH_2NH(CH_3)$	H	Cl	H
N-didesMe-TRM[1]	$CH_2CH_2NH_2$	H	Cl	H
deamino-OH-TRM[1]	CH_2CH_2OH	H	Cl	H
4-OH-TRM[1]	$CH_2CH_2N(CH_3)_2$	OH	Cl	H
4-OH-N-desMe-TRM[1]	$CH_2CH_2NH(CH_3)$	OH	Cl	H
4-OH-deamino-OH-TRM[1]	CH_2CH_2OH	OH	Cl	H

Figure 1. Chemical structures of tamoxifen, toremifene and some metabolites. [1]Berthou and Dreano, 1993; Robinson and Jordan, 1988; [3]Robinson *et al.*, 1991; [4]Ruenitz and Nanavati, 1990; [5]Potter *et al.*, 1994.

Table 1. Summary of biophores used in predictions associated with carcinogenicity of tamoxifen and toremifene.

Database: Biophore	Representative TMX, TRM or metabolite with biophore illustrated	Number of chemicals in database with biophore (active/total)	Probability of activity associated with possession of biophore	Predicted potency range for parent and metabolites
SMDB: HO-NH		4/4	0.83	TMX: 39 TRM: 39
SMDB: -CH$_2$-Cl CPDB: -CH$_2$-Cl		37/48 20/24	0.76 0.91	TMX: not applicable TRM: 35 TMX: not applicable TRM: 50-70
CPDB: CH=CH-C=C-C=		11/13		TMX: 44-116 TRM: 41-114
NTP CDB CH=C-C=C-		5/5	0.86	TMX: 41-52 TRM: 43-56
NTP CDB: C=CH-C=C		35/47	0.74	TMX: 41-54 TRM: 42-54
NTP CDB: O | CH-CH-C=C-CH=		7/7	0.86	TMX: 57 TRM: 57
MCPDB [C-]<--- 6.0 Å --->[OH-]		14/16	0.83	TMX: 52 TRM: 52
RCPDB: [C-]<--- 8.4 Å --->[OH-]		7/7	0.89	TMX: 85 TRM: 85
RCPDB: O-CH3 | =C-C=CH-CH=		5/5	0.86	TMX: +85 TRM: not applicable

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THE CARCINOGENICITY OF DIETHYLSTILBESTROL: STRUCTURAL EVIDENCE

FOR A NON-GENOTOXIC MECHANISM

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SUMMARY

An analysis of the structure of diethylstilbestrol (DES) indicates that neither DES nor any of its metabolites are potential mutagens. Moreover, the present analyses suggest (a) that the observed carcinogenic spectrum of DES reflects the activity of metabolic intermediates and (b) that the carcinogenicity of DES in mice is due to the presence of a 6Å geometric descriptor that appear to be related to an estrogen receptor.

INTRODUCTION

Diethylstilbestrol (DES) is a recognized rodent as well as human cancer-causing agent (reviewed by IARC, 1987a; Marselos and Tomatis, 1992) the mechanism of action of which remains the subject of some controversy. Although DES has been reported to induce a number of cytogenetic effects (IARC, 1987b), its lack of "structural alerts" for DNA-reactivity and its lack of mutagenicity in *Salmonella* as well as its low electronegativity have been taken as evidence that it is a "non-genotoxic" carcinogen (Ashby and Paton, 1993; Ashby and Tennant, 1991). Additionally, a weight of evidence analysis of the results of short-term tests has also concluded that DES is "non-genotoxic" (Rosenkranz et al., 1986). On the other hand, the recent report that exposure of Syrian hamsters to DES results in the formation of DNA "adducts" detectable by the ^{32}P-postlabeling technique, suggests either that these DNA adducts are "I-compounds" (i.e. endogenous adducts) resulting from DES-induced hormonal stress (Liehr et al., 1986) or that indeed DES or one of its metabolites acts by a "genotoxic" mechanism (see Bhat et al., 1994). It is to be noted, however, that using similar procedures, Montandon and Williams (1994) did not find evidence for DES-induced adducts in either treated rats or Syrian hamsters. An understanding of whether DES causes cancer as a result of a genotoxic or a non-genotoxic

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mechanism relates to the extent of the risk to humans of exposure to DES as well as to other estrogens, including xenoestrogens.

Thus, if DES is genotoxic, it could, for example, "initiate" cancer in young women, and subsequent environmental, occupational and/or lifestyle factors might act as promoters. On the other hand, as a non-genotoxicant, DES could act as a "promoter" of previously "initiated" women. The second scenario is considered to present less risk to exposed women than the former.

In order to elucidate these different possibilities, we undertook a study of the potential basis of the carcinogenicity of DES and its metabolites based upon structural features recognized as associated with mutagenicity in *Salmonella* and carcinogenicity in rodents.

MATERIALS AND METHODS

Expert System: CASE/MULTICASE

The CASE methodology has been described on a number of occasions (Klopman, 1984; Klopman and Rosenkranz, 1984). For the present investigation we used the recently developed MULTICASE (MC) program (Klopman, 1992; Klopman and Rosenkranz, 1994). Basically MC selects its own descriptors automatically from a learning set composed of active and inactive molecules. The descriptors are readily recognizable single, continuous structural fragments that are embedded in the complete molecule. The descriptors consist of either activating (biophore) or inactivating (biophobe) fragments. Each of these fragments is associated with a confidence level and a probability of activity which is derived from the distribution of these biophores and biophobes among active and inactive molecules.

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Upon completion of the fragment analyses, MC selects the most important of these fragments as a biophore, i.e. the functionality that is responsible for the experimentally observed activity of the molecules that contain it. MC then selects the molecules containing this biophore for use as a learning set to identify the chemical properties (i.e. structural fragments) or physical chemical properties (e.g. log P, water solubility, quantum mechanical parameter such as HOMO and LUMO, etc.) that modulate the activity of the initially identified biophore. This process generates a QSAR equation for this subset of molecules. If the data set is congeneric, then the single biophore and associated modulators may explain the activity of the entire training set; however, this will usually not occur and there will be a residue of molecules that are not explained by the single biophore and modulators. When this happens, the program will remove from consideration the molecules already explained by the previous biophore and will search for the next biophore and associated modulators. The process continued until the activity of all the molecules of the learning set have been explained.

The list of biophores identified by MC is then used to predict the activity of yet untested molecules. Thus, upon submission for evaluation, MC will determine whether or not an unknown molecule contains an identifiable biophore. In its absence, the molecule will be predicted to be inactive unless it contains a group that resembles chemically one of the biophores, in which case it will be flagged. When the molecule contains a biophore, the presence of modulators for that biophore will be investigated. MC will then make qualitative as well as quantitative predictions of the activity of the unknown molecule.

Obviously, while biophores are the determining structures, the modulators may determine whether and to what extent the biological potential of the chemical is expressed.

Additionally, MC incorporates selection rules to identify two-dimensional distance descriptors based upon the presence of lipophilic centers. Initially, heteroatoms and lipophilic carbon atoms are designated as "special" atoms. A carbon atom is designated as a lipophilic center if it is at least four bonds away from a heteroatom and it is also the furthest carbon away from the heteroatom when its neighbors are considered. After all the "special" atoms are identified the distances between all possible pairs is calculated. The distribution of these descriptors among active and inactive molecules is analyzed for statistical significance. Various atom groupings are also investigated, i.e., hydrogen bond acceptors and donors as well as halogens.

Expert System: META

The expert system "META", a computer based metabolism program, was employed to generate putative metabolites of DES. The META program has been recently described in detail (Klopman *et al.*, 1994; Talafous *et al.*, 1994). META contains a knowledge set of 665 enzyme-catalyzed reaction rules including most of the phase I and II enzyme reactions and 286 spontaneous reactions. When presented with the structure of a parent molecule, META describes a series of metabolic pathways.

For this investigation DES was "metabolized" by META through what can be visualized as four branches, i.e., DES was metabolized through one iteration which yielded four metabolites; two of the metabolites were unconjugated to sulfonic or glucuronic acids and were metabolized for four additional metabolic iterations.

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DATABASES

Salmonella mutagenicity database

The National Toxicology Program (NTP) SMDB was generated under the aegis of the of the U.S. NTP (Ashby and Tennant, 1991; Cater *et al.*, 1986; Haworth *et al.*, 1983, 1989; Lawlor *et al.*, 1985; Mortelmans *et al.*, 1984, 1986; Zeiger, 1987, 1990; Zeiger and Haworth, 1985; Zeiger *et al.*, 1985, 1987, 1988). The data base consists of 1354 chemicals of which 482 are mutagens, 26 are marginal mutagens and 846 are non-mutagens.

Rodent carcinogenicity database

The rodent carcinogenicity data base was also generated under the aegis of the U.S. NTP (summarized in Ashby and Tennant, 1991). In that database, chemicals of known purity were tested under coded conditions in a standardized two year bioassay. Chemicals were tested at the maximum tolerated dose (MTD) in addition to lower doses. After serial sacrifices or at the termination of the bioassays, complete gross and microscopic analyses of the rodent tissues were performed. The interpretation of the results were reviewed by an external panel of experts.

For the purpose of the present analyses we used the summaries of the bioassays on 301 chemicals (Ashby and Tennant, 1991). Due to previously described limitations (Rosenkranz and Klopman, 1990), only 287 chemicals were suitable for analysis by MC. Subsequently, due to the ambiguous nature of chemicals classified as equivocal carcinogens, these were also removed from the database. This left a total of 255 chemicals for analysis.

The chemicals that were subjected to analyses were assigned potency values in CASE units. These values are not related to the dose needed to induce cancer, i.e., in contrast to the

TD_{50} values (see below). Rather they reflect the carcinogenic spectrum, i.e., from trans-species carcinogens to carcinogens active in only a single tissue of a single sex of a single species. The classification and CASE unit designations are as follows:

A: Agents carcinogenic to rats and mice at one or more sites.

- Assigned 60 CASE units.

B: Agents carcinogenic only to the rat or mouse at two or more sites.

- Assigned 50 CASE units.

C: Agents carcinogenic only to the rat or mouse at a single site in both sexes

- Assigned 40 CASE units.

D: Agents carcinogenic at only a single site in a single sex of a single species.

- Assigned 30 CASE units.

E: Agents adequately tested for which equivocal evidence of carcinogenicity was obtained.

- Assigned 20 CASE units; deleted from database.

NC: Agents adequately tested and concluded to be non-carcinogenic

- Assigned 10 CASE units.

Carcinogenic Potency Database

The carcinogenic potency database was assembled by Gold *et al.* (1984, 1986, 1987, 1990, 1993). Two subsets were derived from this compilation (rodent and mouse). In contrast to the NTP rodent carcinogenicity data base, this compilation is based primarily on published reports. Presumably these were not subjected to the rigorous quality assurance and peer review

process of the NTP. However this database has two advantages with respect to the present study:

a. For chemicals judged to be carcinogenic, the dose required for 50% of the animals to remain cancer free is calculated (TD_{50}, which accounts for spontaneous cancer) (Gold *et al.*, 1984; Peto *et al.*, 1984). This provides a more characteristic measure of potency, i.e., the amount of chemical needed to induce cancer in a species.

b. The number of chemicals included in this database is greater than that included in the NTP data base. This results in an increased informational content of the database (Rosenkranz and Klopman, 1991; Takehi *et al.*, 1993) resulting in substantial refinements in the nature of the structural determinants.

For each database, all reported dosages were transformed into gavage equivalents. Additionally, the TD_{50} value in mg/kg/day were converted into mmol/kg/day. Using the proper equations (below) chemicals were assigned to activity groups. Chemicals in the range of 10 to 19 CASE units are inactive or exhibited negligible activity. Chemicals with activities in the range of 20 to 29 CASE units are marginally active and chemicals in the range of 30 to 99 CASE units are carcinogenic.

The rodent and mouse carcinogenic potency databases relevant to each MC analysis are discussed below in greater detail.

(a) Rodent carcinogenic potency database

The rodent CPDB consists of 437 chemicals, 265 of which are active, 8 are marginally active and 164 are inactive. To be included in this database, a chemical had to have been tested in both rats and mice. To designate potencies in CASE units, the chemicals reported by the

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authors to be non-carcinogenic in rodents were assigned 10 CASE units. In addition, those chemicals with a TD_{50} value in excess of 28 mmol/kg/day were added to this category. If the chemical was found to be carcinogenic in both rats and mice the value for the more sensitive species, i.e., the lower TD_{50} value, was used.

For the purpose of the SAR analyses, TD_{50} values (i.e. potencies) in mmol/kg/day were transformed into CASE units using the following relationship:

$$\text{CASE units} = 18.3279 * \log 1 / TD_{50} + 46.5517 \qquad \text{(Equation 1)}$$

Using Equation 1, chemicals in the range of 10 to 19 CASE units are inactive or exhibit negligible activity. Chemicals with activities in the range of 20 to 29 CASE units are marginally active and chemicals in the range of 30 to 99 CASE units are carcinogenic.

(b) Mouse carcinogenic potency database

The mouse CPDB consists of 639 chemicals, 291 of which are active, 11 are marginal and 337 are non-carcinogenic. Chemicals reported by the authors to be non-carcinogenic in mice were assigned 10 CASE units along with chemical with TD_{50} value in excess of 51 mmol/kg/day.

For the purpose of the SAR analyses TD_{50} values (i.e. potencies) in mmol/kg/day were transformed into CASE activity units using the following relationshipp:

$$\text{CASE activity} = 14.1329 * \log 1 / TD_{50} + 44.1329 \qquad \text{(Equation 2)}$$

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Using Equation 2, chemicals in the range of 10 to 19 CASE units are inactive or exhibit negligible activity. Chemicals with activities in the range of 20 to 29 CASE units are marginally active and chemicals in the range of 30 to 99 CASE units are carcinogenic.

RESULTS AND DISCUSSION

Fifteen metabolites of DES were identified from published reports and another 126 putative metabolites were generated by the expert program META[1]. Based upon the *Salmonella* mutagenicity database, neither DES nor any of its known or putative metabolites were predicted to be mutagens (Cunningham and Rosenkranz, 1994). Thus, DES metabolism presumably does not result in DNA-reactive intermediates, suggesting that DES is non-genotoxic. It should be noted, however, that a similar study of the metabolites of tamoxifen led to the identification of putative metabolites that are mutagenic (Cunningham and Rosenkranz, 1995).

Examination of the parent molecule, DES, in a variety of carcinogenicity data bases led to the prediction that DES is a rodent carcinogen. Thus, based upon the NTP Rodent Carcinogenicity Data Base, DES is predicted to be carcinogenic (Figure 1) by virtue of the presence of biophore A which originates from five carcinogenic molecules present in the data base ($p = 0.03$). The predicted "potency" (41 CASE units), indicates (see above) that the chemical has a potential for being carcinogenic to single species at multiple sites in both sexes.

Based upon CPDB, DES is also predicted to be a rodent carcinogen. This prediction is based upon biophore B (Figure 2) which, in fact, is an elaboration of biophore A derived from the NTP data base. Biophore B is present in 13 molecules, 11 of which are carcinogens ($p =$

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[1] The details of the META analysis and a list of the metabolites are available to readers from Cunningham and Rosenkranz (1994).

0.01). The projected activity of 97 CASE units for biophore B is equal to a TD_{50} value of 0.57 mmoles/kg/day.

The CPDB analysis also revealed that the majority of DES metabolites are predicted to be carcinogenic by virtue of the presence of biophore B, and moreover, the projected TD_{50} values remain in the same range as that calculated for DES (Figure 2).

Similarly, based upon the NTP data base, the majority of the DES metabolites are predicted to be carcinogenic. However, a number of metabolites were projected to have increased "potencies" (as expressed in the projected CASE units) which, in the instance of the NTP data base, suggests a broadened carcinogenic spectrum. Thus, the known metabolite 3'3"-dimethoxy-E-diethylstilbestrol (Figure 3) and the META generated putative metabolite S23 (Figure 4) are projected to have potencies of 57 CASE units, thereby indicating a potential for inducing cancers in mice and rats at multiple sites (see above). Indeed, this is the characteristic carcinogenic spectrum of DES (IARC, 1987a). These results indicate that the trans-species carcinogenic activity associated with DES, may, in fact, be derived from some of its metabolites.

Analysis of DES and its metabolites using the mouse CPDB resulted in the prediction that DES is carcinogenic by virtue of the presence of a geometric descriptor of 6.0 Å (Figure 5). The 6.0 Å geometric descriptor has also been associated with estrogenicity and anti-estrogenicity (unpublished results). This biophore could possibly represent a ligand for an estrogen binding site. Most of the metabolites of DES retain this descriptor (Cunningham and Rosenkranz, 1994).

The present study suggests that neither DES nor any of the metabolites are "genotoxicants" as judged by their potential to induce mutagenicity in *Salmonella*. Moreover, it should be noted that one of the putative DES metabolites, i.e. DES-2,3,-oxide is predicted by CASE to be non-mutagenic despite the presence of the epoxide, a putative "structural alert" for

DNA-reactivity (Ashby and Tennant, 1991). In fact, this lack of mutagenicity has been confirmed experimentally (Glatt et al., 1979). The present study reinforces the notion that DES is carcinogenic by virtue of a non-genotoxic mechanism.

Based upon the SAR model described herein, we have demonstrated that many estrogens and anti-estrogens contain the 6.0 Å geometric descriptors found in DES (unpublished results). Thus, it could well be that this biophore identifies the structural basis of the carcinogenicity of DES and of other chemicals which contain it (e.g. ethynylestradiol) thus providing direct structural evidence that the carcinogenicity exhibited by these non-genotoxic agents derives from their estrogenicity. The present study also establishes that the observed carcinogenic spectrum of DES is probably a reflection of the activity of its metabolites as evidenced by the fact that, while the parent molecule is predicted to have a narrow spectrum of carcinogenic activities, its metabolites are predicted to have a much broader activity. This, in fact, reflects the results of animal bioassays (IARC, 1987a).

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Diethylstilbestrol

The molecule contains the biophore (number of occurrences = 4):
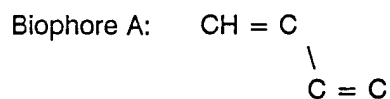
Biophore A: CH = C
                      \
                      C = C

    5 out of the known 5 molecules (100%) containing such biophore are carcinogens with an average activity of 50. (conf.level=97%)

                                                    Constant is 38.7

The following modulator is also present:

    Water solubility = -1.22;   WS contribution is 2.8

The probability that this molecule is a NTP Rodent Carcinogen is 85.7%

The compound is predicted to be very active
The projected carcinogenic spectrum is 41 CASE units
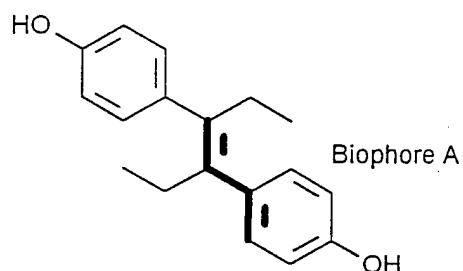
Biophore A

Figure 1. MultiCASE predictions of the carcinogenicity of DES based upon the National Toxicology Program Rodent Carcinogenicity Database. The biophore (A) associated with rodent carcinogenicity is shown in bold. It is derived from five carcinogenic molecules in the data base (C.I. basic red no. 9; polybrominated biphenyl; tetrachlorvinphos; dichlorodiphenyldichloroethylene; 2-biphenylamine HCl).

Diethylstilbestrol

The molecules contains the biophore (number of occurrences = 4):
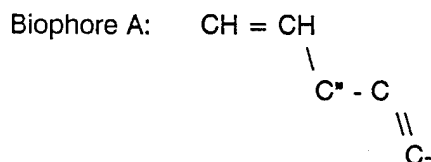
Biophore A: CH = CH
                       \
                      C* - C
                             \\
                              C-

11 out of the known 13 molecules (85%) containing such biophore are carcinogens with an average activity of 50. (conf.level=99%)

Constant is 61.0

The following modulators are also present:
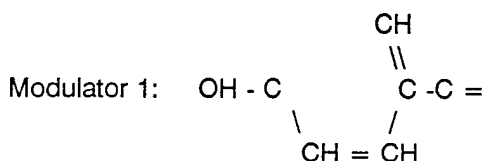
                           CH
                            \\
Modulator 1:   OH - C   C -C =
                        \   /
                         CH = CH   Activating 39.5

    Water solubility = -1.22   WS contribution is -3.5

The probability that this molecule is a CPDB carcinogen is 80.0%

The compound is predicted to be extremely active
The projected carcinogenicity is 97 CASE units
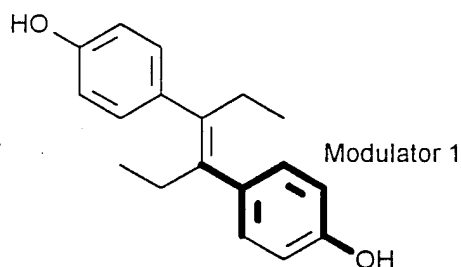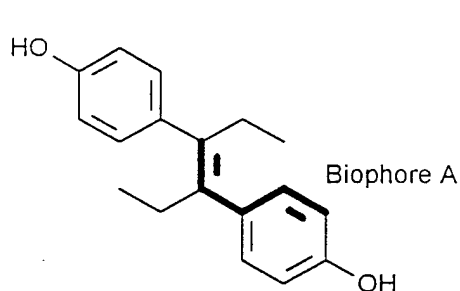
Figure 2. MultiCASE prediction of carcinogenicity of DES based upon the Carcinogen Potency Data Base of Gold *et al.* (1984, 1986, 1987, 1990, 1993) (CPDB). The probability is derived from the fact that biophore A is present in 13 chemicals in the data base, 11 of which are carcinogens. The presence of biophore A is associated with 61 CASE units of potency. The presence of four copies of modulator 2 contributes a further 39.5 CASE units to the potency. The projected potency (97 CASE units) corresponds to a TD_{50} of 0.57 mmoles/kg/day.

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3',3"-Dimethoxy-E-diethylstilbestrol

The molecule contains the biophore (number of occurrences = 2):
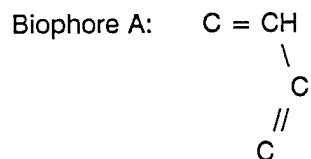
Biophore A: C = CH
                   \
                    C
                   //
                  C

    35 out of the known 47 molecules (74%) containing such biophore are rodent carcinogens with an average activity of 38 (conf.level = 100%)

Constant = 56.4

The following modulator is also present:

    Water solubility = -0.26   WS contribution = 0.6

The probability that this molecule is a rodent carcinogen is 73.5%
The carcinogenic spectrum is projected to be 57 CASE units
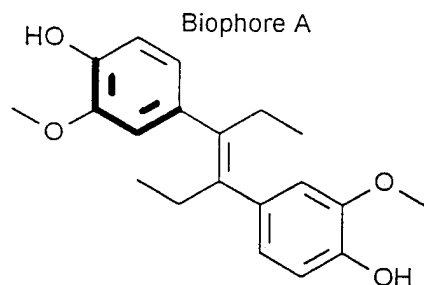
Figure 3. MultiCASE prediction for 3',3"-dimethoxy-E-diethylstilbestrol based on the NTP rodent carcinogenicity database. A spectrum of 57 CASE units indicates a potential for inducing cancers in multiple species at multiple sites in both sexes.

18

Metabolite s23

The molecules contains the biophore (number of occurrences = 2):
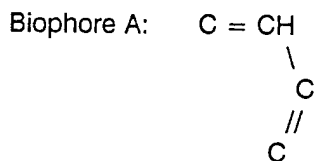
Biophore A: C = CH
                      \
                       C
                      //
                       C

        35 out of the known 47 molecules (74%) containing such a biophore
        are rodent carcinogens with an average activity of 38. (conf.level=100%)

                                                        Constant is 56.4
The following modulator is also present:

        Water solubility = 0.44   ws contribution is -1.0

The molecules also contain the biophore (number of occurrences = 1):

Biophore B: CH = CH
                      \
                       C - O
                      //
              =CH - C
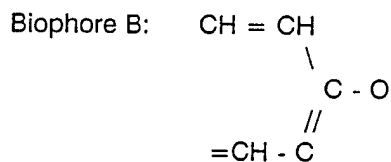
        7 out of the known 7 molecules (100%) containing such a biophore
        are NTP CDB carcinogens with an average activity of 51. (conf.level=99%)

The probability that this molecule is a Rodent Carcinogen is 73.5% due to the first biophore, increased
to 86.3% due to presence of the extra biophore.

The activity is projected to be 57 CASE units
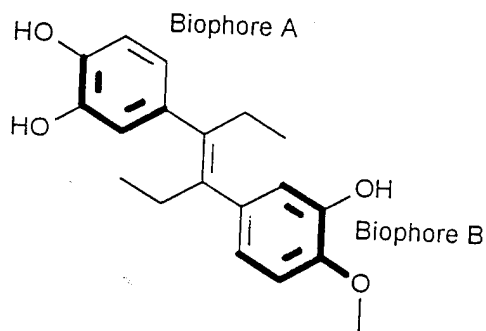
Figure 4. MultiCASE prediction of the carcinogenicity in rodents of metabolite s23.

Diethylstilbestrol

The molecule contains the biophore:

Biophore: 2D fragment [C-]   <--- 6.0 Å ---> [OH-]

14 out of the known 16 molecules (87%) containing such biophore are mouse carcinogens with an average activity of 47 (conf.level = 100%)

Constant = 51.8

The probability that this molecule is a mouse carcinogen is 83.3%

The activity is projected to be 52 CASE units
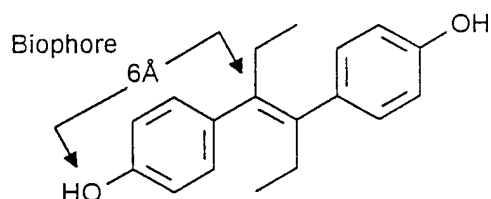
Figure 5. MultiCASE prediction for diethylstilbestrol in the mouse carcinogenic potency database.

STD1 (MSN3) interacts directly with the TATA-binding protein and modulates transcription of the *SUC2* gene of *Saccharomyces cerevisiae*

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ABSTRACT

STD1 (MSN3) was isolated independently as a multicopy suppressor of mutations in the TATA-binding protein and in SNF4, suggesting that STD1 might couple the SNF1 kinase signaling pathway to the transcriptional machinery. We report here a direct physical interaction between STD1 and the TATA-binding protein (TBP), observed *in vivo* by the two-hybrid system and *in vitro* by binding studies. STD1 bound both native TBP in yeast cell-free extracts and purified recombinant TBP. This interaction was altered when TBPΔ57 was used, suggesting a role for the non-conserved N-terminal domain of TBP in mediating protein–protein interactions. We also show that perturbation of STD1-TBP stoichiometry alters *SUC2* expression *in vivo* and that this effect is dependent on the N-terminal domain of TBP. The activation of *SUC2* expression by increased copy number of *STD1* occurs at the level of mRNA accumulation and it requires the same TATA element and uses the same transcription start site as does activation of *SUC2* by glucose limitation. Taken together, these results suggest that STD1 modulates *SUC2* transcription through direct interactions with TBP.

INTRODUCTION

STD1 was isolated as a multicopy suppressor of growth defects caused by overexpression of the conserved C-terminal domain of the TATA-binding protein (TBPΔ57) (1). The TATA-binding protein (TBP) is an essential component of the transcriptional machinery of all three nuclear RNA polymerases (2–6). In yeast, TBP contains 240 residues with the C-terminal 180 residues consisting of a DNA binding domain that is highly conserved throughout eukaryotic evolution. The 60 residue N-terminal domain is not conserved and is not required for viability (7–10). Overexpression of wild type TBP in yeast has no known

phenotype. In contrast, overexpression of TBPΔ57 from the *GAL1* promoter can result in a dominant negative phenotype of extremely slow growth and defects in both induced and uninduced RNA polymerase II transcription (1,11). This effect is not restricted to growth on galactose media; overexpression of TBPΔ57 (y183c) from the *ADH1* promoter causes poor growth on media with glycerol as a carbon source (8).

Biochemical studies indicate that the N-terminal domain inhibits the DNA binding activity of TBP. The temperature dependence of TBP's DNA binding activity is relaxed by proteolytic removal of the N-terminal domain (12). The N-terminal domain of TBP greatly destabilizes the TBP–DNA complex in electrophoretic mobility shift assays and it increases the activation energy of TBP–DNA complex formation by almost 3 kcal/mol (13). Missense mutations that reduce DNA binding activity of TBP are suppressed *in vitro* by removal of the N-terminal domain (14). These same mutant forms of TBP can bind DNA stably if TFIIA is present, indicating that TFIIA may stabilize the DNA bound form of TBP by blocking the inhibitory effects of the N-terminal domain (14). These data suggest that the N-terminal domain negatively affects the DNA binding activity of TBP and that other proteins may be able to modulate this effect. Since TBPΔ57 causes deleterious effects only when expressed at high levels, TBPΔ57 might be titrating an important component of the transcription machinery.

By searching for multicopy suppressors of TBPΔ57 overexpression, we sought to identify genes encoding proteins that directly interact with or regulate the activity of TBP. *STD1* on a multicopy plasmid suppresses both the growth phenotype and the defects in RNA polymerase II transcription caused by overexpression of TBPΔ57 (1). It does this even though TBPΔ57 accumulates to the same levels and has the same DNA-binding activity as without *STD1* overexpression. High level expression of *STD1* suppresses TBPΔ57 induced defects in transcription at apparently unrelated loci such as *CUP1* and *ACT1*. These findings suggest that STD1 may be an important component of transcription, perhaps interacting directly with the transcription machinery.

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STD1 (MSN3) was also identified as a multicopy suppressor of the raffinose growth defect caused by deletion of the *SNF4* gene (15). Deletion of *SNF4* causes a defect in the SNF1 protein kinase pathway of glucose derepression (16–18). Strains with this defect have pleiotropic phenotypes related to carbon utilization (19–22). Among other things, these strains fail to derepress transcription of *SUC2*, the gene for invertase, and as a result, cannot grow on media containing raffinose as the carbon source.

Several lines of evidence indicate that STD1 acts positively to derepress invertase expression (15). First, increased copy number of *STD1* restores the ability of a *snf4Δ* strain to derepress invertase and grow on raffinose media. Secondly, increased copy number of *STD1* partially relieves glucose repression in wild type cells. Thirdly, deletion of *STD1* and its homologue, *MTH1*, causes a 4-fold reduction in the derepressed level of invertase expression. These activities are all dependent upon an intact *SNF1* gene. Moreover, STD1 can physically associate with SNF1 *in vivo* and *in vitro*. These data suggest that STD1 acts in conjunction with SNF1 to relieve glucose repression of the invertase gene.

STD1 does not appear to be a conventional transcriptional activator. A LexA-STD1 fusion protein fails to activate transcription of a reporter gene containing LexA binding sites (15). This fusion protein is known to be functionally active, since it suppresses the *snf4Δ* phenotype. Sequence analysis of STD1 does not detect any similarity to motifs associated with known families of transcription factors (1,15). Thus the mechanism by which STD1 acts to derepress invertase expression is unclear.

We report here a direct physical interaction between STD1 and TBP. We show that *in vivo*, STD1 interacts with TBP in the two-hybrid system. *In vitro*, purified recombinant STD1 binds both native TBP from yeast whole cell extracts and recombinant TBP purified from *Escherichia coli*. STD1 is also able to bind TBPΔ57, though in a qualitatively different fashion from its binding to TBP. *In vivo*, the expression of *SUC2* is sensitive to perturbations of STD1-TBP stoichiometry and this effect is also dependent on the N-terminal domain of TBP. These results suggest that STD1 activates expression of invertase through its interaction with TBP at *SUC2* and that this interaction is modulated by the N-terminus of TBP.

MATERIALS AND METHODS

Strains and genetic methods

Saccharomyces cerevisiae strain Y153 (*MATa, leu2-3,112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δ, gal80Δ, URA3::GAL1-lacZ, LYS2::GAL1-HIS3*) was used for the two-hybrid analysis (23). Y153 requires GAL4 activity to grow on media lacking histidine. MCY2634 (*MATa, snf4-Δ2, ura3-52, his3-Δ200, leu2-3,112*) was used for testing invertase expression in a *snf4Δ* background (15). MCY2662 (*MATa, ura3-52, trp1-Δ1, his3-Δ200, lys2-801*) was used to prepare yeast extracts to test the ability of GST-STD1 to bind TBP. MCY2649 (*MATα, ura3-52, leu2-3, 112, his3Δ200*) and FY716 (*MATα, ura3-52, leu2Δ1, his4-912δ, lys2-128δ, suc2-104*) were used in invertase assays. Yeast were grown in synthetic complete (SC) media (24), lacking certain supplements where indicated to maintain plasmid selection. Dextrose was used as the carbon source unless otherwise noted.

Plasmid constructions

Plasmids pGBT9 and pGAD424 were used to construct fusions for two-hybrid analysis (25). pGBT9 expresses the GAL4 DNA binding domain (amino acids 1–147) from the *ADH1* promoter, while pGAD424 expresses the GAL4 activation domain (amino acids 768–881) from the *ADH1* promoter. In both cases the test protein is fused to the C-terminal end of the *GAL4* fragment. The GAL4 binding domain-STD1 fusion (pTT21) was created using PCR with 5'-CCGGAATTCATGTTTGTTTCACCACCTCC and 5'-CGCGAATTCAAATTTACTAGGACATTCCATCAGGCTT-CC as primers to amplify a fragment containing the entire *STD1* gene with *Eco*RI sites on each end, in frame with the *GAL4* binding domain fragment in pGBT9. This PCR product was subcloned into the *Eco*RI site of pGBT9 to make pTT21. The GAL4 activation domain-TBP fusion plasmid (pTT31) was similarly constructed except a threonine to lysine mutation was introduced at residue 112 to abolish the DNA binding activity of TBP (10). The mutation was created using the primers 5'-GCGCGAATTCATGGCCGATGAGGAAC with 5'-GCAA-AAATTAAAGCTTTAGTTTTTGG and 5'-CCAAAAACTAA-AGCTTTAATTTTTGC with the universal primer. The resulting two overlapping PCR products were used with the two outside primers in a new PCR reaction to generate full length TBP with the T112K mutation. This PCR product was digested with *Eco*RI and *Sal*I and inserted into similarly digested pGAD424 to create pTT31. The T112K mutation and the fusion junctions were confirmed by DNA sequence analysis.

Plasmid pGEX-STD1 was constructed using PCR to amplify the full length STD1 using the primers 5'-CGCGGATCCCA-TATGTTTGTTTCACCACCTCC and 5'-CGCGAATTCAAA-TTTACTAGGACATTCCA. The resulting PCR product was digested with *Bam*HI and *Eco*RI ligated into similarly digested pGEX-2T (26).

Plasmid pRG84 was constructed by subcloning the 2.4 kb *Eco*RI-*Bam*HI genomic fragment containing the wild type TBP gene, *SPT15*, into similarly digested YEp352 (27). pRG85 is the same as pRG84 with a precise deletion removing amino acids 2–57 in TBP.

Plasmid pGBT9-SNF1 (the GAL4 binding domain-SNF1 fusion) was subcloned from pEE5 (28), pGBT9-LAM (the GAL4 binding domain–human lamin C fusion)was subcloned from pLAM5 (29). pNI12 (28) (the GAL4 activation domain fused to the C-terminus of SNF4), pGST-MSN3 (15) (a glutathione S-transferase-STD1 fusion protein; GST-STD1), pLexA (1–202)+PL (30), pLexA-MSN3 (15) (the LexA binding domain-STD1 fusion) and pDE93-3 (31) (the 2μ vector pRS424 expressing TBP from its native promoter) have all been described previously.

Two-hybrid assays

For the two-hybrid assays (28,32), yeast strain Y153 was co-transformed with the indicated pGBT9 and pGAD424 derivatives onto synthetic complete media lacking tryptophan and leucine to maintain selection for the plasmids. Transformed cells were grown in selective media to late log phase and adjusted to equal cell number. Ten microliter each of 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions were spotted to synthetic complete media with 2% ethanol, 2% galactose and 3% glycerol as a carbon source and lacking tryptophan, leucine and histidine. 3-Aminotriazole (3-AT) was added at 30 mM to increase the requirement for the *HIS3* gene

product (23). These dilutions were also spotted to the same media containing histidine to control for equal viability. Cells were grown for 7 days at 30°C, then photographed.

Assay for invertase activity in *snf4Δ* strains

Strain MCY2634 was transformed with pLexA(1–202)+PL or pLexA-MSN3 and either YEp352, pRG84 or pRG85. Glucose derepressed cells were prepared (21) and assayed for invertase activity (33) .

Primer extension of *SUC2* mRNA

Total yeast RNA was prepared (34) and analyzed by primer extension (35) using AMV reverse transcriptase. The oligonucleotide primer complementary to *SUC2* mRNA (5′-CCAAAGGTC-TATCGCTAGTTTCGTTTGTCATTGATGCAGATATTTTGG-CTGC) was labeled with [γ-^{32}P]ATP and polynucleotide kinase.

Assay for binding to GST-STD1

Glutathione S-transferase-STD1 was purified as previously described (15) from *E.coli* strain XL1-blue (Stratagene) transformed with pGST-MSN3 or pGEX-STD1, for the experiments shown in Figures 2 and 3, respectively. To test the ability of GST-STD1 to bind TBP in yeast whole cell extracts, MCY2662 transformed with pDE93-3 (expressing TBP from a multicopy plasmid) was grown to mid-log phase in selective media containing 2% dextrose (repressing). Cells were then shifted to media containing 0.05% dextrose (derepressing) for 3 h. Yeast whole cell extracts were then prepared (16) and 200 μg whole cell extract per assay was incubated with GST or GST-STD1 resin. The resin was washed 10-fold with 1 ml of MTPBS (15), boiled in SDS-PAGE sample buffer and analyzed by 10% SDS-PAGE (36). Western analysis was performed by standard methods (36), using rabbit anti-TBP serum (kindly provided by S. Buratowski, Whitehead Institute) and developed using a chemiluminescent method (Amersham).

For testing the ability of STD1 to bind purified recombinant TBP and TBPΔ57, GST-STD1 bound to glutathione-agarose was incubated in 100 mM NaCl, 1% Triton X100, 20 mM NaPO$_4$ pH 7.3 in the presence of protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 1 μg/ml each aprotinin, leupeptin and pepstatin) for 30 min at 4°C with 2 μg of either purified recombinant TBP or TBPΔ57. This was centrifuged and the supernatant was saved. The resin was washed six times in the same buffer. One half of the resin was then eluted with SDS-PAGE sample buffer. The other half was subjected to sequential salt washes containing 0.15, 0.2, 0.4 and 0.8 M NaCl in 1% Triton X100, 20 mM NaPO$_4$, pH 7.3 and protease inhibitors as indicated above. This was followed by a final wash with a buffer containing 20 mM glutathione, 0.12 M NaCl, 100 mM Tris, pH 8.0. The remaining resin was then eluted with SDS-PAGE sample buffer. The GST samples were treated identically, except that the bound GST was eluted directly with glutathione buffer. These samples were then analyzed by SDS-PAGE in a 12% gel. Western analysis was by standard methods (36). A polyclonal rabbit anti-TBP antibody was used to visualize TBP. TBPΔ57 was visualized by a polyclonal rabbit antibody directed against the C-terminus of TBP (1). The blots were developed using an alkaline phosphatase method (Biorad).
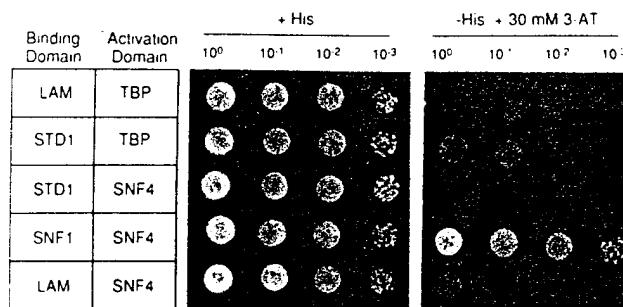

Figure 1. STD1 interacts with TBP *in vivo*. Strain Y153 transformed with the indicated plasmids were grown overnight in selective media to late log phase. They were adjusted to equal cell number, then 10 μl of the indicated dilutions were spotted onto synthetic complete media lacking tryptophan and leucine or to media lacking tryptophan, leucine and histidine and containing 30 mM 3-AT. They were grown for 7 days at 30°C, then photographed.

RESULTS

STD1 and TBP interact in the two-hybrid system

The ability of STD1 to suppress TBPΔ57-induced defects in a dosage dependent manner suggested that STD1 may interact directly with TBP. We tested this possibility using the two-hybrid system developed by Fields and colleagues (28,32). This assay is based on the fact that GAL4 activation of a reporter gene can be reconstituted in *trans* from separate fusion proteins containing the GAL4 binding (G$_{BD}$) and activation (G$_{AD}$) domains. Reconstitution of GAL4 activity *in vivo* indicates a physical interaction between the two fusion proteins.

Initial experiments determined that a fusion protein composed of wild type yeast TBP and G$_{BD}$ contained significant background activity in the absence of any G$_{AD}$ plasmid. This technical difficulty was overcome by making two adjustments. We used a point mutation in the DNA binding domain of TBP (Thr to Lys at position 112) that eliminates specific binding to TATA boxes (10) and we fused this (TBP$_{T112K}$) to G$_{AD}$. The G$_{AD}$-TBP$_{T112K}$ fusion contained little if any background activity and was used in the two hybrid experiments described below.

Plasmids expressing G$_{BD}$ fused with STD1 (G$_{BD}$-STD1) and G$_{AD}$ fused with TBP$_{T112K}$ (G$_{AD}$-TBP$_{T112K}$) were cotransformed into the yeast strain Y153. Y153 has its *HIS3* gene under the control of the *GAL1* upstream activating sequence and the endogenous *GAL4* gene is deleted. Reconstitution of GAL4 activity from the fusion proteins allows expression of the *HIS3* gene product and the resulting ability to grow on media lacking histidine. 3-Aminotriazole was added to the media to increase the amount of the *HIS3* gene product required, since the background level of transcription that occurs in the absence of any GAL4 activity is sufficient for substantial growth (23). Thus, increasing amounts of 3-AT can be used to titrate the efficiency of GAL4 reconstitution.

Cells containing both G$_{BD}$-STD1 and G$_{AD}$-TBP$_{T112K}$ were able to reconstitute GAL4 activity, demonstrating an interaction between STD1 and TBP. This is shown for serial dilutions of these strains at 30 mM 3-AT in Figure 1, on media containing ethanol, glycerol and galactose as a carbon source. Similar results were obtained with 15 mM 3-AT and on glucose with 15, 30 and 60 mM 3-AT (not shown).
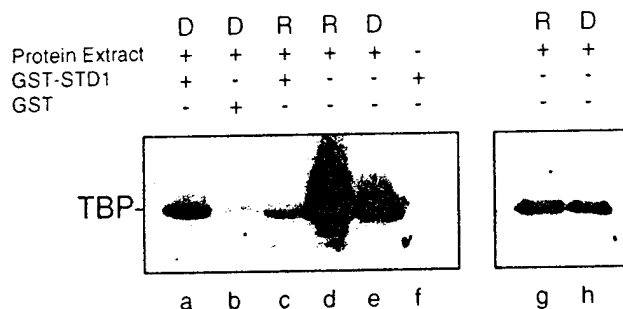
Figure 2. Glutathione S-transferase-STD1 binds TBP from yeast whole cell extracts. Strain MCY2662 transformed with pDE93-3 (TBP expressed from a 2 µ vector) was grown to mid-log phase in selective media containing 2% dextrose (repressing, R) and shifted to derepressing (D) media for 3 h. Yeast extracts were prepared and 200 µg protein from each was incubated with GST-STD1 (lanes a and c) or GST (lane b) bound to glutathione-agarose for 1 h at 4°C. After extensive washing, these were boiled in SDS-PAGE sample buffer and subjected to SDS-PAGE on a 10% gel. 25 µg yeast extract was loaded in lanes d and e. Lane f was loaded with GST-STD1 alone. Lanes g and h are the same as d and e, except at a lighter exposure. TBP was detected by Western analysis using rabbit anti-TBP serum and developed by chemiluminescence.

The STD-TBP interaction was shown to be specific, in that neither G_{BD}-STD1 nor G_{AD}-TBP$_{T112K}$ promoted growth when paired with other proteins. For instance, while the G_{BD}-LAM (human lamin C)/G_{AD}-TBP$_{T112K}$, G_{BD}-STD1/SNF4-G_{AD} and G_{BD}-LAM/SNF4-G_{AD} protein pairs showed some growth at the 10^0 dilution, it was not sustained. At the 10^{-1} dilution these strains were clearly not growing. The positive control, the G_{BD}-SNF1/SNF4-G_{AD} pair, shows a well documented (28,37), relatively strong interaction. The interaction between TBP and STD1 was not dependent on the T112K mutation since similar results were obtained with wild type TBP fused to either the LexA or GAL4 DNA binding domains (data not shown). That the same results were obtained using different constructs and reporter genes shows that the apparent ability of G_{BD}-STD1 and G_{AD}-TBP$_{T112K}$ to interact was not an artifact of any particular construct. Furthermore, all strains grew on media supplemented with histidine (Fig. 1), indicating that none of the fusion proteins were toxic. Thus, G_{BD}-STD1 and G_{AD}-TBP$_{T112K}$ were able to reconstitute GAL4 activity through their specific interaction *in vivo*.

STD1 specifically binds TBP from yeast extracts

The two hybrid data reported above provided evidence that STD1 and TBP interact *in vivo*. We detected this interaction *in vitro* using a GST-STD1 purified from bacteria. Glutathione S-transferase-STD1 or GST bound to glutathione-agarose resin was incubated with 200 µg yeast extract from either glucose repressed or derepressed cells. After extensive washing, the proteins bound to the resin were analyzed by Western blot. GST-STD1 specifically bound TBP (Fig. 2, compare lanes a and e; c and d). Glutathione S-transferase did not (lane b). This biochemical evidence confirms that STD1 specifically interacts with TBP in both glucose repressed and derepressed cells.

STD1 binds purified recombinant TBP and TBPΔ57

We addressed whether STD1 interacts with TBP directly or through an intermediate by testing the ability of GST-STD1 to bind recombinant TBP purified from *E.coli*. We also tested its ability to
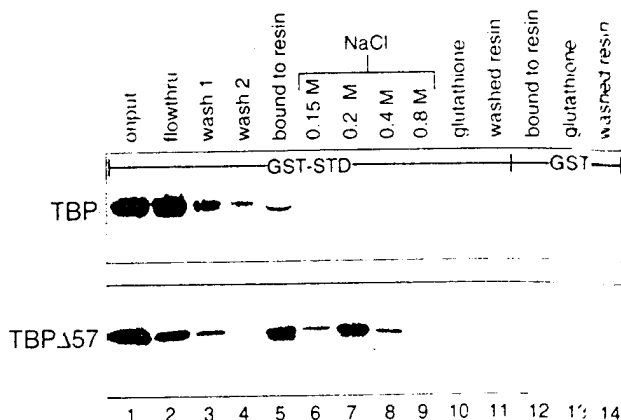
Figure 3. Glutathione S-transferase-STD1 directly interacts with TBP and TBPΔ57. Freshly induced GST-STD1 was bound to glutathione-agarose; then incubated in isolation buffer at 0.1 M NaCl for 30 min at 4°C with either purified recombinant TBP or TBPΔ57 (input). This was centrifuged and the supernatant was saved (flowthru). The resin was washed three times in the same buffer (wash 1), then three more times (wash 2). One half the resin was then eluted with SDS-PAGE sample buffer (bound to resin). The other half was subjected to sequential salt washes in isolation buffer containing 0.15, 0.2, 0.4 and 0.8 M NaCl. Bound proteins were then eluted from the resin with a buffer containing 20 mM glutathione, 0.12 M NaCl, 100 mM Tris, pH 8.0 (glutathione). Any remaining bound proteins were then eluted by boiling with SDS-PAGE sample buffer (washed resin). The GST samples were treated identically, except that one half the resin was eluted with glutathione buffer immediately after wash 2.

bind TBPΔ57, since an increased gene dosage of STD1 can rescue cells from TBPΔ57 toxicity. Glutathione S-transferase-STD1 or GST bound to glutathione-agarose resin was incubated with 2 µg purified recombinant TBP or TBPΔ57. The resin was then washed and eluted with increasing concentrations of NaCl. Figure 3 shows a Western analysis of these fractions using antibodies to TBP and TBPΔ57. Both TBP and TBPΔ57 bound to GST-STD1 (lane 5), but not to GST alone (lane 12). This interaction was not mediated by DNA, since including either ethidium bromide (38) or DNase I in these reactions had no effect relative to controls (data not shown). These results demonstrate a direct physical interaction between STD1 and TBP or TBPΔ57 proteins *in vitro*.

Interestingly, there were striking qualitative and quantitative differences in the binding properties of TBP and TBPΔ57. The same preparation of GST-STD1 resin bound almost half the TBPΔ57, but only a small fraction of the TBP (compare lanes 2 and 5). This difference was not due to different amounts of active TBP or TBPΔ57 in these preparations, since they have similar DNA binding activities as measured by electrophoretic mobility shift assay (data not shown). Furthermore, the binding of STD1 to TBP appeared qualitatively different from that of STD1 to TBPΔ57. Most of the bound TBPΔ57 eluted at 0.2 M NaCl (lane 7), whereas the small amount of TBP that bound either eluted at 0.15 M NaCl or was retained until the glutathione wash. These experiments show that both TBP and TBPΔ57 interact with STD1, but that removal of the non-conserved N-terminal domain of TBP alters its binding properties to STD1.

STD1 activation of *SUC2* does not alter TATA box or start site selection

Since STD1 protein interacts with the TBP, it is possible that its mechanism of activation of the *SUC2* gene may be to direct TBP

to an alternative TATA element. Additional TATA-like sequences are present at −32 (TATAT) and −121 (TATAAT) relative to the mRNA start site and are capable of binding TBP *in vitro* (data not shown). Utilization of these TATA elements might also result in an alternative start site of transcription initiation. Therefore, we investigated the TATA box and start site utilization at *SUC2* when derepressed by STD1 or by glucose limitation. For these experiments, we utilized the *suc2-104* mutation which replaces the −90 TATA element (TATAAA) with a KpnI restriction site (39). Cells carrying the wild type *SUC2* or the *suc2-104* allele were compared for their ability to derepress invertase expression in response to glucose limitation and in response to increased gene dosage of *STD1*. In wild type cells, glucose limitation resulted in a 50-fold increase in invertase enzyme activity. Increased copy number of the *STD1* gene resulted in a 10-fold increase of invertase expression even though cells were maintained under repressing (high glucose) conditions (Fig. 4A). These results are consistent with earlier studies (15,39). In contrast, the *suc2-104* mutation essentially blocks the ability of cells to derepress *SUC2* expression in response to either glucose limitation or increased copy number of *STD1*. This strain (FY716) has all the *trans*-acting factors needed for regulation of *SUC2* since they efficiently derepress wild type *SUC2* introduced on a plasmid (not shown). These data demonstrate that STD1 activation of *SUC2* depends on the same TATA element that is used in response to glucose limitation.

The initiation site of *SUC2* mRNA was analyzed by primer extension of total RNA using a *SUC2* specific oligonucleotide primer. Using this assay, we compared both the quantity of *SUC2* mRNA and the initiation site selection. Wild type cells show a large increase in *SUC2* mRNA in response to glucose limitation (Fig. 4B, lanes 2 and 5) that closely correlates with the observed increase in invertase activity (Fig. 4A). The mobility of the primer extension product is consistent with the previous mapping of the *SUC2* mRNA start site to 40 bp upstream of the ATG codon (40). Increased copy number of *STD1* increases *SUC2* mRNA accumulation under repressing conditions (lane 3) and the mobility of the primer extension product is identical to that observed for *SUC2* mRNA induced by glucose limitation. No additional primer extension products were observed. Therefore, STD1-activated mRNA has the same 5′ end as mRNA derepressed by glucose limitation. In addition, we examined the level of *SUC2* mRNA in cells which lack both the *STD1* gene and its homologue, *MTH1*. These cells derepress invertase poorly (15) and this defect is apparent at the level of mRNA accumulation (lane 4).

TBP can titrate STD1 suppression of the snf4Δ phenotype

Given that STD1 can interact directly with TBP, we sought evidence that this interaction is relevant *in vivo*. Previously, it was shown that STD1 expressed from a multicopy plasmid can partially suppress the defect in invertase expression of a *snf4Δ* strain (15). To test whether this ability of STD1 to suppress the *snf4Δ* defect is mediated by its interaction with TBP, we measured the invertase activity of strains overexpressing both STD1 and TBP.

For this experiment a LexA-STD1 fusion protein under the control of the strong *ADH1* promoter was used. The LexA moiety is not relevant here. As expected from previous studies (15), LexA-STD1 restored significant invertase activity in a *snf4Δ* strain under derepressing conditions and that function was dependent on the STD1 moiety in the fusion protein (Fig. 5, lanes
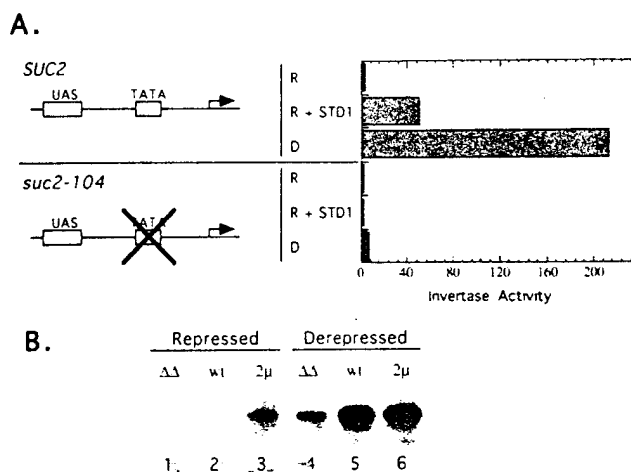
A.

B.

Figure 4. *SUC2* promoter elements required for activation by STD1 and glucose limitation. (A) TATA box requirement. The requirement for the *SUC2* promoter TATA element was examined by comparing strains MCY2649 (*SUC2⁺*) and FY716 (*suc2-104*) for the ability to derepress *SUC2* transcription in response to glucose starvation and increased copy number of *STD1*. All cells were transformed with the YEp13 based plasmid (2μ, *LEU2*) and grown in the absence of leucine. Cells transformed with YEp13 are indicated by R and D; cells transformed with p6A8, a plasmid with the *STD1* gene inserted into YEp13 are indicated by R + STD1. Repressed cells (R and R + STD1) were grown in synthetic complete media containing 2% glucose to an OD of 0.5–1.0, and were then harvested and assayed for invertase activity. Derepressed cells (D) were grown to an OD of 0.5–1.0 in the same media and were then harvested, washed in water and resuspended to the same OD in the same media containing 0.05% glucose. After 3 h, cells were harvested and assayed for invertase activity. All errors were <5% for values >2 U/100 mg dry weight. (B) Primer extension of *SUC2* mRNA. The cells used in this experiment contained wild type levels of STD1 and MTH1 (wt), increased copy number of *STD1* on a 2μ plasmid (2μ) or contained null alleles of both *std1* and *mth1* (ΔΔ). Total yeast RNA was purified from cells grown under repressing conditions (2% glucose: lanes 1–3) or derepressing conditions (3 h in 0.05% glucose: lanes 4–6). Primer extension products from reactions with 15 μg of RNA were resolved on an 8% polyacrylamide, 7 M urea gel. Control reactions indicated that this analysis was in the linear range for this assay (data not shown). A reaction with twice the RNA (30 μg) from wild type derepressed cells yielded twice the quantity of extension product, whereas a 2-fold increase in primer did not increase the yield of primer extension product.

a and d). Increased dosage of the TBP gene on a multicopy plasmid abolished STD1 suppression of *snf4Δ* (lane e). In control experiments, the presence of the parent vector for the TBP plasmid had little effect (lane d), as did increased dosage of TBP in the absence of overexpressed STD1 (lane b). Instead, high levels of TBP seem specifically to interfere with STD1 suppression of the *snf4Δ* defect. In contrast, increased dosage of TBPΔ57 on a multicopy plasmid had little effect on invertase expression in the *snf4Δ* strain, whether LexA-STD1 was present (lane f) or not (lane c). These results are consistent with a direct interaction between TBP and STD1 that is modulated by the N-terminus and further suggest that this interaction is physiologically relevant to the role of STD1 in the regulation of *SUC2* transcription.

DISCUSSION

The isolation of *STD1* (*MSN3*) as a multicopy suppressor of both a *snf4Δ* mutant and a TBP mutant suggests that this protein might
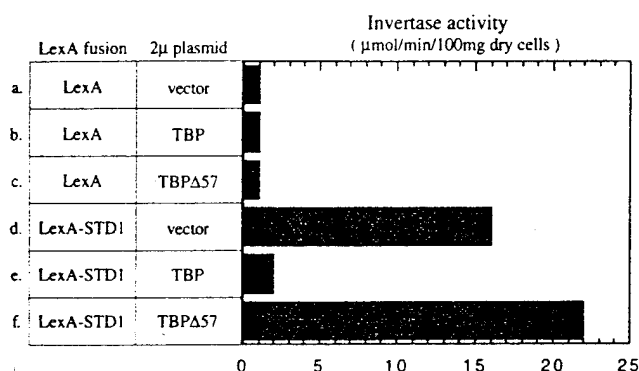
	LexA fusion	2μ plasmid	Invertase activity (μmol/min/100mg dry cells)
a.	LexA	vector	
b.	LexA	TBP	
c.	LexA	TBPΔ57	
d.	LexA-STD1	vector	
e.	LexA-STD1	TBP	
f.	LexA-STD1	TBPΔ57	

0   5   10   15   20   25

Figure 5. High copy expression of TBP abolishes STD1 suppression of *snf4Δ* phenotype. Strain MCY2634 transformed with either pLexA(1–202)+PL (LexA) or pLexA-MSN3 (LexA-STD1) and either YEp352 (vector), pRG84 (TBP) or pRG85 (TBPΔ57) were grown to mid log phase in selective media and shifted to glucose derepressing media (0.05% glucose) for 2 h. Yeast cells were prepared and tested for invertase activity. A unit is defined as micromoles of glucose released per min/100 mg (dry weight) of cells. Values are the average of assays of three transformants for derepressed samples. All errors were <8% for values >2 U/100 mg dry weight.

provide a link between the SNF1/SNF4 protein kinase complex and the transcriptional machinery. Earlier studies demonstrated a physical interaction between STD1 and SNF1, both *in vivo* and *in vitro* (15). Here we demonstrate a direct physical interaction between STD1 and TBP, thus establishing a potential link between SNF1 and the transcriptional machinery.

Interaction of STD1 and TBP

In vivo, STD1 interacted with TBP in the two-hybrid system. This interaction was shown to be specific and independent of particular plasmid constructs or yeast strains. The interaction between STD1 and TBP was also demonstrated *in vitro* using a GST-STD1 fusion protein. When purified GST-STD1 was incubated with extracts from yeast cells, it was shown to bind TBP. This interaction was specific in that GST alone did not bind TBP. The interaction of TBP with GST-STD1 was also detected using purified components, showing that this interaction is direct and not dependent on additional yeast proteins. Together with the two-hybrid results, these data provide conclusive evidence for a direct physical interaction between STD1 and TBP.

Many gene regulatory proteins have been shown to interact with TBP (41–50). In fact, so many TBP-interacting proteins have now been identified that it is reasonable to question which interactions are biologically important. The data presented here indicate that the STD1-TBP interaction is physiologically relevant *in vivo*. Changes in the relative levels of expression of STD1 and TBP or TBPΔ57 cause changes in gene regulation *in vivo* (1,15). For instance, increased expression of TBPΔ57 causes defects in RNA polymerase II transcription *in vivo* that can be reversed by increased expression of STD1. Similarly, increased expression of STD1 causes a partial derepression of *SUC2* in a *snf4Δ* mutant that can be reversed by increased expression of TBP. In both cases, the effects on transcription by overexpressing one of these proteins is reversed by concomitant overexpression of the other. The dosage effects of these

phenotypes are consistent with the existence of a STD1-TBP interaction that affects transcriptional regulation *in vivo*.

The mechanism by which the STD1-TBP interaction might affect transcriptional regulation remains speculative. The phenotypes due to TBPΔ57 overexpression and *snf4Δ* mutation correlate with defects in gene expression. Increased copy number of *STD1* can partially suppress both these phenotypes and the data presented here and previously (15) provide a physical link between the components of glucose derepression and general transcription. However, the data do not provide a clear mechanistic model that can adequately explain our *in vitro* binding data. In particular, it is not known whether a STD1-TBP complex is the active species with regard to *SUC2* transcription or whether these proteins affect *SUC2* transcription by titrating one or the other from a different complex. A second issue is the identity of the component of this system which provides gene specificity. The mechanism which directs these components to the *SUC2* gene as opposed to any other TATA-box-containing gene remains unknown.

Role of the N-terminal domain of TBP

Our data indicate that the interaction of STD1 and TBP is altered by the non-conserved N-terminal domain of TBP. This domain of TBP is not essential *in vivo* and little is known about its functional role. *In vitro*, the N-terminal domain acts as an inhibitor of DNA binding (12,13,51) and this inhibition can be modulated by TFIIA (14). The binding studies using GST-STD1 with purified TBP and TBPΔ57 show that the STD1-TBP interaction is greatly affected by the N-terminal domain of TBP, providing evidence for a role of the N-terminal domain of TBP in modulating protein–protein interactions. The N-terminal domain of TBP also affects the STD1-TBP interaction *in vivo*. Increased expression of STD1 results in increased expression of *SUC2* in a *snf4* mutant. This STD1-mediated derepression is blocked by increased expression of TBP but not TBPΔ57. The finding that the N-terminal domain affects the STD1-TBP interaction may explain earlier studies which reported that cells expressing TBPΔ57 (y183C) grew poorly on some carbon sources (8). Deletion of both *STD1* and its homologue, *MTH1*, also results in poor growth on these media (15). Recently, it was found that the TBPΔ57 but not full-length TBP was able to associate with human TAFs when expressed in a human cell line, suggesting that the N-terminal domain may affect the assembly of the TFIID complex *in vivo* (52). These data suggest that one role of the N-terminal domain may be to modulate the interaction of TBP with the TAFs and other transcriptional regulators.

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THE YEAST SWI/SNF COMPLEX BINDS TO DNA AND HAS BINDING PROPERTIES SIMILAR TO HMG-BOX DOMAINS.

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The SWI/SNF complex is required for the enhancement of transcription by many transcriptional activators in yeast[1,2]. Both genetic and biochemical studies provide evidence that the complex facilitates activator function by using the energy of ATP hydrolysis to antagonise chromatin-mediated transcriptional repression[3-7]. Here we show that the SWI/SNF complex has a high affinity for DNA and that the DNA binding properties of the complex are highly reminiscent of those exhibited by HMG-box domains[8]. For example, we demonstrate that the complex binds to DNA with low sequence specificity, interacts with the minor groove of the DNA helix, binds synthetic 4-way junction DNA, and introduces supercoils into relaxed plasmid DNA. These HMG-box properties are likely to play a major role in the mechanism by which the SWI/SNF complex remodels chromatin structure.

The yeast SWI/SNF complex is comprised of 11 subunits[7,9,10], five of which were identified in early genetic screens as the SWI1, SWI2/SNF2, SWI3, SNF5 and SNF6 polypeptides[1]. The absence of known DNA binding motifs in these known SWI/SNF subunits, together with unsuccessful attempts to identify SWI/SNF-dependent DNA binding activities in crude yeast extracts, has led to the belief that the complex does not bind DNA[11,12]. Recently we reported the purification of the yeast SWI/SNF complex to near homogeneity[7], thus we sought to assay the *purified* complex for DNA binding activity. Our initial experimental strategy was to assay binding of the purified complex to DNA fragments by gel retardation analysis. Figure 1A illustrates that the complex binds with high affinity (kD = 3 x 10^{-9} M) to promoter sequences from both SWI-dependent (*SUC2* and *ADH2*) and SWI-independent (*CYC1*) genes. We

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also found that the complex bound equally well to both promoter and coding region sequences derived from the SWI-dependent gene, *HO*, thus illustrating that the complex has no intrinsic affinity for regulatory sequences (data not shown). In all DNA binding experiments the addition of ATP had no effect on the formation of protein-DNA complexes (data not shown). Although the complex does appear rather promiscuous for high affinity DNA binding, it has some sequence specificity. For instance, all binding reactions contain a 1000-fold molar excess of the competitor DNA poly(dG.dC). In addition, the complex binds poorly to the upstream activation sequence of the SWI-dependent *INO1* gene (Fig. 1A) and the 154 bp GAL4 binding sequence used in our previous study[7] (data not shown). Furthermore, figure 2A illustrates that binding of the complex to ADH2 and CYC1 DNA probes resulted in protection of specific sequences from DNase I. However, a comparison of the SWI/SNF binding sites revealed no apparent sequence similarities and no consensus footprint size (Table 1). As in the DNA binding assays, addition of ATP to footprinting reactions had no effect (data not shown).

Three observations indicate that this DNA binding activity is due to SWI/SNF complex. First, SWI/SNF complex can be fractionated on a native DNA cellulose column which increases the purity of the SWI/SNF preparations (see legend, Fig. 1). Secondly, DNA binding activity co-elutes with SWI/SNF complex from the final gel filtration column (data not shown). Thirdly, addition of a monoclonal antibody directed against an engineered epitope at the C-terminus of SWI2 disrupts and supershifts the DNA-protein complex (Fig. 1B, lane 3). Addition of an irrelevant monoclonal antibody had no effect (Fig. 1B, lane 2). UV-crosslinking experiments were performed to identify which of the 11 polypeptides of the SWI/SNF complex contact DNA. Three

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polypeptides were consistently cross-linked corresponding in size to the 150 kDa SWI1 polypeptide and to two (p68 and p78) components of the complex that have not been cloned as yet (Fig. 1C). One possibility is that only one of these subunits directly contacts DNA. Alternatively, all three subunits of the SWI/SNF complex may exhibit DNA binding properties.

We have performed a rigorous examination of the DNA binding properties of the SWI/SNF complex and found that the complex shares many properties characteristic of HMG-box domains. Like several HMG-box containing polypeptides[8], the SWI/SNF complex does not appear to recognise a consensus DNA sequence (Table 1). Minor groove binding drugs, distamycin A (Fig. 3A) and chromomycin A3 (data not shown), compete with the SWI/SNF complex for DNA binding suggesting that, like HMG-box proteins [13,14,15], the complex interacts (at least in part) with the minor groove. We also found that the SWI/SNF complex, like the HMG-box domain containing polypeptides HMG-1[16] and UBF [17], exhibited a DNA length dependence in binding assays (Fig. 3B).

One striking feature of HMG-box domains is their ability to recognise irregular DNA structures[8]. Bianchi and colleagues originally identified HMG-1 as a major nuclear protein that recognised cruciform DNA[18], and subsequently other HMG-box containing polypeptides have been shown to bind to structured DNAs[14,19]. As illustrated in Figure 3C, the SWI/SNF complex also binds with high affinity to synthetic cruciform DNA but not to the duplex "arms" of the cruciform. This result suggests that the SWI/SNF complex may recognise structural features of DNA rather than the primary DNA sequence. Interestingly, the four-way junction structure formed in the synthetic

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cruciform DNA may mimic the cross-over point where DNA enters and exits the nucleosome[20].

We also found that the SWI/SNF complex can supercoil circular DNA in the presence of bacterial topoisomerase I (Fig 4A). The complex did not introduce supercoils in the absence of topoisomerase I, nor did this activity require ATP. The observation that eukaryotic topoisomerases were able to remove the SWI/SNF-induced supercoils (Fig.4B, compare lanes 6 to 8 & 9) whereas *E. coli* topoisomerase I had no effect (Fig. 4B, compare lanes 6 and 7) indicates that the SWI/SNF complex introduces exclusively positive supercoils. Several HMG-box containing polypeptides, including HMG-1[21], mtTF1[22] and ABF2[23] have previously been shown to induce negative supercoils into closed circular DNA in the presence of topoisomerase I. What is the basis of the SWI/SNF complex-induced supercoiling? One possibility is that the SWI/SNF complex wraps the DNA template in a right-handed direction thus introducing positive supercoils. Alternatively, the complex may use the energy of DNA binding to directly change the helical twist, resulting in an overwinding of the DNA. Such a change in helical twist would be incompatible with nucleosome stability, thus it is tempting to speculate that the SWI/SNF complex may use such an activity to disrupt nucleosomal DNA[7].

One prediction of the DNA wrapping model is that the positive supercoils will be constrained by the SWI/SNF complex; and thus addition of an eukaryotic topoisomerase during the supercoiling assay will not inhibit induction of supercoils. On the other hand, if SWI/SNF does not constrain the positive supercoils, for instance as predicted by the above "twisting" model, then a eukaryotic topoisomerase will block the SWI/SNF induced supercoiling

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reaction. As shown in Figure 4C, calf thymus topoisomerase I inhibits the induction of supercoils by SWI/SNF (compare lanes 2 and 3 to lanes 4 and 5). This inhibition is specific to the supercoiling activity of SWI/SNF, as calf thymus topoisomerase I did not block the binding of SWI/SNF to the synthetic cruciform DNA in a gel shift assay (data not shown). Thus the inability of calf thymus topoisomerase I to support SWI/SNF-induced supercoiling is inconsistent with the wrapping model, and suggests that the complex may induce supercoiling by changing the helical twist of DNA.

The HMG-box domain properties of the SWI/SNF complex are likely to be central to the mechanism of SWI/SNF action. For example, mutations in SIN1, an HMG 1-like polypeptide in yeast, alleviates the need for a functional SWI/SNF complex[3]. The data presented in this paper suggest that the complex may function, at least in part, by competing with SIN1 and other HMG-like proteins for DNA binding. Furthermore, the ability of the SWI/SNF complex to modulate both DNA structure and topology may play a major role in SWI/SNF-dependent disruption of nucleosome structure or function.

A

SWI/SNF ← | SUC2-1 | SUC2-2 | SUC2-3 | INO1 |
| ADH2-1 | ADH2-2 | ADH2-3 | CYC1 |

SWI/SNF ← (ADH2-1, ADH2-2, ADH2-3, CYC1)

B

| Control | anti-FLAG | anti-HA |

1 2 3

C
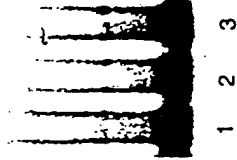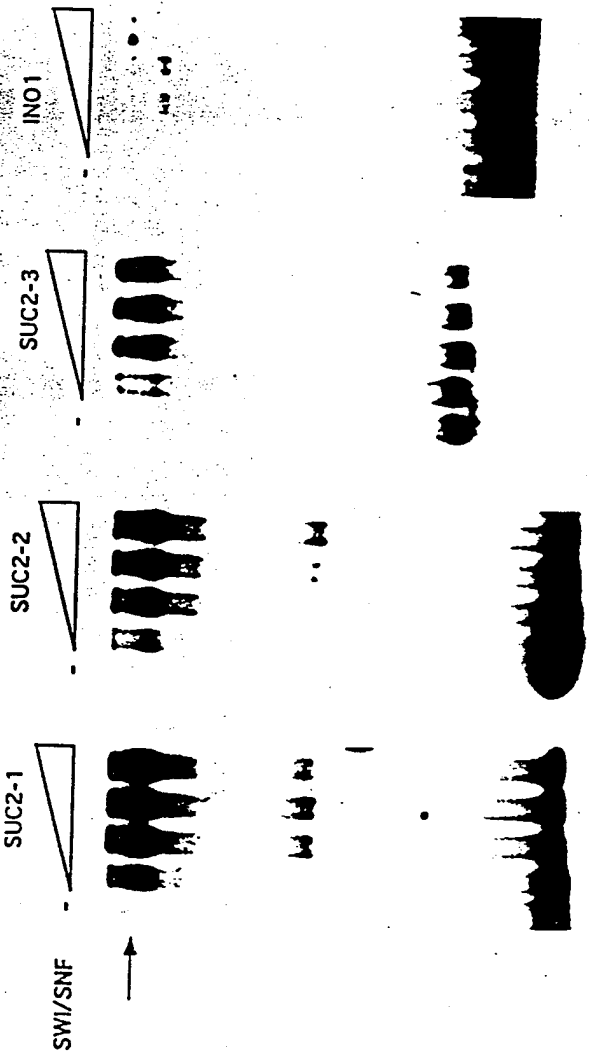
Competitor:	-	-	-	-	+
X-linking:	-	+	+	+	+
SWI/SNF:	-	-	+	+	+

200
97.4
68
43
25.7
18.4

1 2 3 4 5
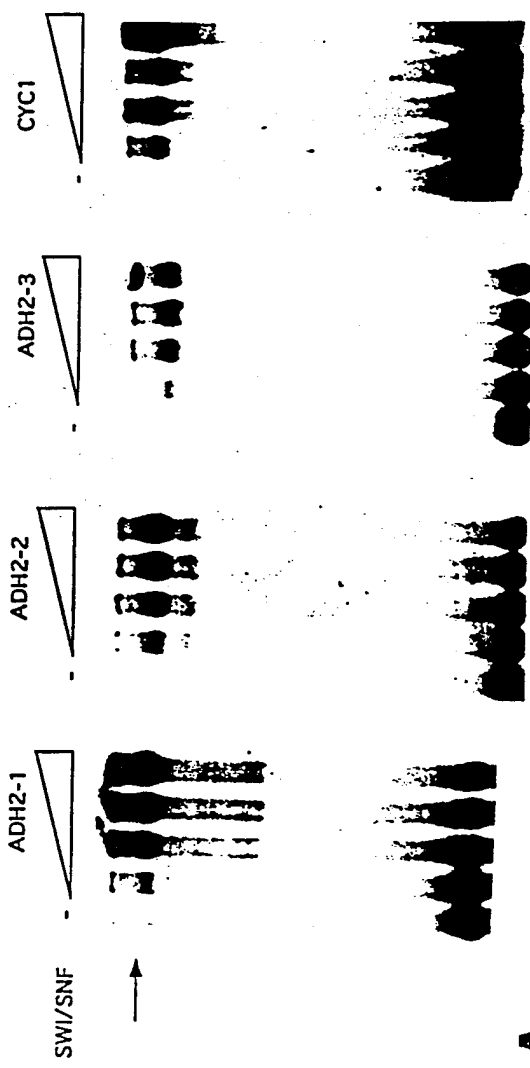
FIGURE LEGENDS

FIGURE 1. The yeast SWI/SNF complex binds to DNA.

Panel A. Gel retardation analysis. Each panel shows a set of parallel gel retardation experiments in which increasing amounts of highly purified SWI/SNF complex (1.5 to 6.0 nM) were incubated with 0.1 nM of ^{32}P-labelled promoter sequences derived from both SWI-dependent (SUC2, ADH2, INO1) and SWI-independent genes (CYC1). Probes used in these experiments include: (1) SUC2-1, SUC2-2 and SUC2-3 which contain bases -171 to +14, -171 to -386 and -386 to -712 respectively of the SUC2 promoter, (2) the INO1 probe which encompasses bases -126 to -333 of the INO1 promoter, (3) ADH2-1, ADH2-2 and ADH2-3 which contain bases -413 to -182, -182 to -54, and -54 to +116 respectively of the ADH2 promoter, (4) the CYC1 fragment which encompasses bases -249 to +11 of the CYC1 promoter. Panel B. Antibody supershifts. DNA binding reactions, that contained 0.1nM of the SUC2-1 fragment and 1nM SWI/SNF, were supplemented with 80 ng of anti-FLAG monoclonal antibody (lane 2), 80 ng of the 12CA5 monoclonal antibody (lane 3) or received no antibody addition (lane 1). Panel C. UV-cross linking of the SWI/SNF complex to the SUC2-3 probe. Lane 1, no UV cross-linking; lane 2, no SWI/SNF; lane 3, crosslinking of the SWI/SNF complex to the SUC2-3 sequence; lane 4, crosslinking of the complex in the presence of a 20-fold molar excess of unlabelled SUC2-3 fragment. All reactions contained 6nM SWI/SNF complex and 0.1 nM SUC2-3 fragment.

Methods. DNA binding assays were performed in 15 µl reaction mixtures containing DNA binding buffer (4mM Tris-Cl, pH 7.4, 5mM $MgCl_2$, 75mM NaCl, 1mM DTT, 0.05mg/ml BSA, 4% glycerol), 300ng of poly(dG-dC) competitor DNA and 0.1 nM of end labelled ^{32}P-probe DNA. The SWI/SNF complex was

7

CYC 1   ADH2-2   ADH2-1

SWI/SNF —   —   —

-119
-162

-131

+77

-198

-158

+97

-221
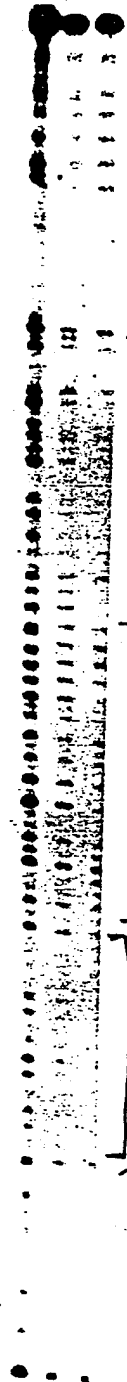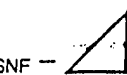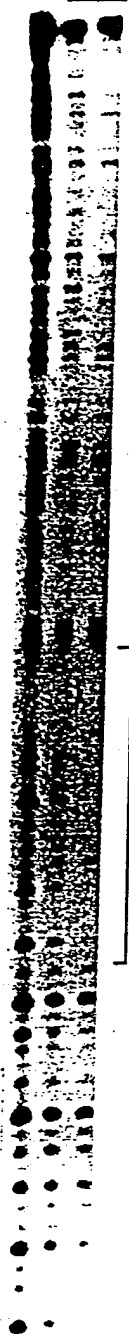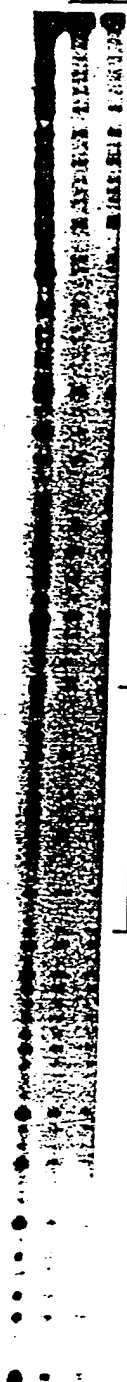
added last, to give final concentrations between 1.5 and 6 nM, and reactions were incubated for 30 minutes at room temperature. Protein-DNA complexes were resolved on 4.5% polyacrylamide (80 acrylamide:1 bis-acrylamide ratio)/ 0.4x TBE gels, and analysed by autoradiography. *The SWI/SNF complex was purified* essentially as previously described[7], with the addition of a DNA cellulose column. Peak fractions from the Mono Q step (in 350 mM NaCl) were diluted to 100mM NaCl and loaded onto a 0.5ml DNA cellulose column pre-equilibrated in DNA binding buffer. Bound protein was eluted with DNA binding buffer containing 300mM NaCl, and then subjected to gel filtration as described previously[7]. This additional step resulted in SWI/SNF preparations that lacked contaminating polypeptides, as analysed by SDS-PAGE. *UV-crosslinking experiments.* The SUC2-3 probe was internally labelled with $[\alpha^{32}P]$dCTP, and bromodeoxyuridine was incorporated in place of dTTP to increase the efficiency of cross-linking. Standard DNA binding reactions contained 0.1 nM of probe DNA and 6nM of the purified SWI/SNF complex. Reaction mixtures were UV-irradiated as described previously[24], and analysed on 10% SDS-polyacrylamide gels.

FIGURE 2. Footprinting analysis of the yeast SWI/SNF complex.
DNAse I digestion patterns of the CYC1, ADH2-1 and ADH2-2 probes, either in the absence (lanes 1, 4 & 7) or in the presence of 6nM (lanes 2, 5 & 8) and 12nM (lanes 3, 6 & 9) SWI/SNF complex. *Methods.* Gel retardation assays were scaled up by a factor of 2, to which 0.2 nM of probe DNA was added and various amounts of the SWI/SNF complex as described above. Binding reactions were incubated for 30 minutes, followed by DNAse I digestion (0.03 U) for 1 minute at room temperature. Reactions were stopped by the addition of 5 volumes of STOP (5mM Tris, pH 7.5, 175mM NaCl, 10mM EDTA, 3.5M

8

Distamycin — — ▱ (gradient)
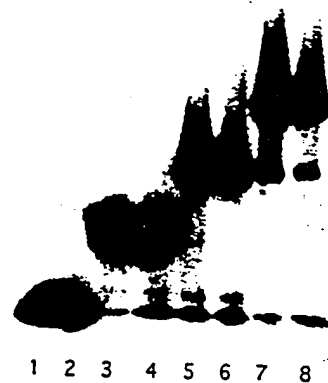SWI/SNF − + + + + +

A.
1 2 3 4 5 6
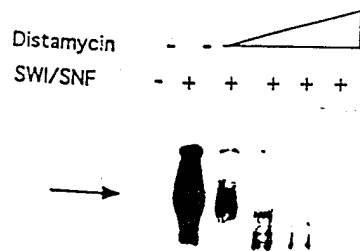
Probe 30 bp 80bp 134 bp 179 bp
SWI/SNF − + − + − + − +

B.
1 2 3 4 5 6 7 8

C.

Duplex 1   Duplex 2

4 WJ

D.
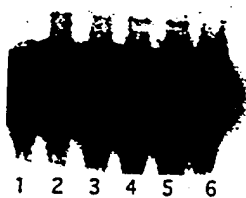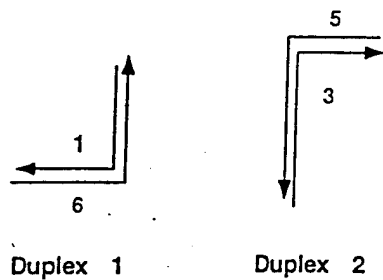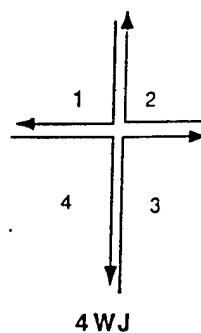
SWI/ SNF   4WJ   Duplex 1   Duplex 2

1 2 3 4   5 6 7 8 9 10 11 12
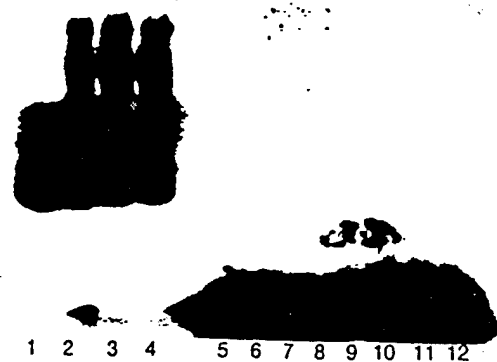
urea, 1% SDS), phenol/chloroform extracted and ethanol precipitated. Samples were analysed on 8% polyacrylamide/8M urea sequencing gels.

**FIGURE 3. The SWI/SNF complex has similar DNA-binding properties to HMG box domains.**

Panel A. The minor groove binding reagent distamycin A competes with the SWI/SNF complex for binding to DNA. Increasing amounts of distamycin were added to DNA binding reactions, that contained 0.1nM labelled SUC2-3 fragment and 3nM SWI/SNF complex, and its effect on binding assessed by gel mobility shift analysis. Lane 1: probe DNA alone, lane 2: probe DNA and the SWI/SNF complex, lanes 3-6 contain a constant amount of the SWI/SNF complex with increasing amounts of distamycin A. Concentrations used were; lane 3: 0.1 μM (molar ratio of reagent per bp = 0.4), lane 4: 1μM (molar ratio = 4), lane 5: 10μM (molar ratio = 40), lane 6: 100μM (molar ratio 400). **Panel B.** The SWI/SNF complex exhibits a length dependence upon binding to DNA. The complex (3nM) was tested for binding to the -129 to -158 sequence of the ADH2 promoter to which we had previously observed footprinting (lane 2), an 80mer (lane 4), an 134 mer (lane 6), and an 179mer (lane 8) all containing the -129 to -158 sequence. All DNA probes were present at 0.1nM in the binding reactions. **Panels C and D.** The SWI/SNF complex binds to cruciform DNA. Panel C shows a schematic representation of the synthetic cruciform DNA and of the control duplex DNAs. For the sequence of the oligonucleotides see ref. 25. Oligonucleotides 1-4 are partially complementary to each other and, when annealed, assemble to form a four way junction (4WJ) DNA structure. Oligonucleotides 5 & 6 are complementary to oligonucleotides 3 and 1 respectively. On annealing they form perfectly

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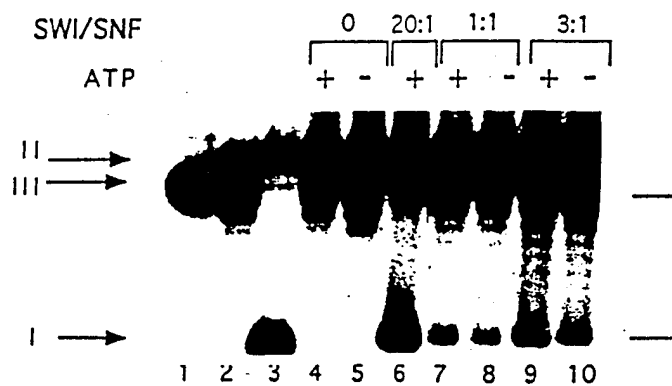
A.
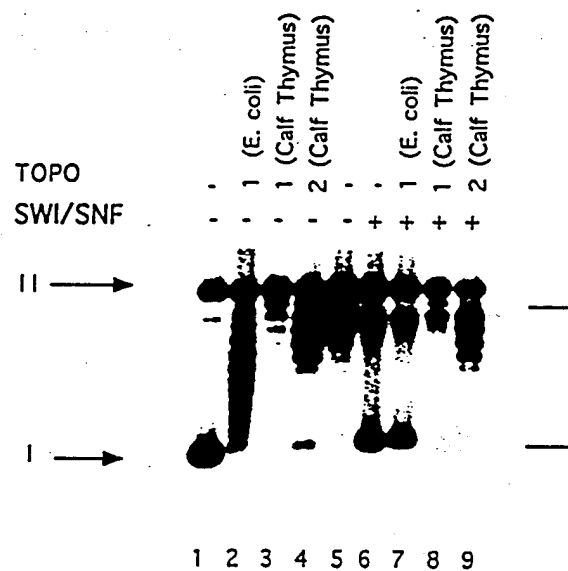
B.
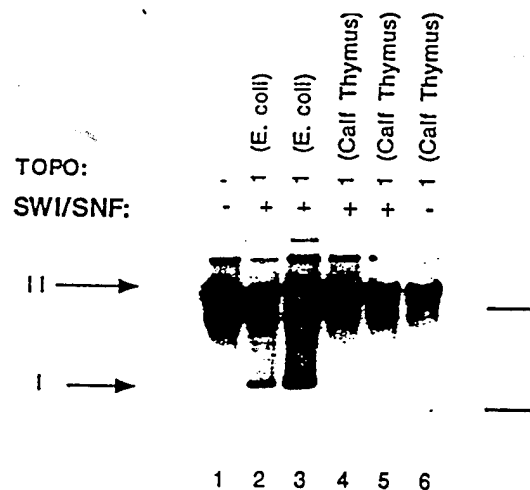
duplex molecules whose sequences are the same as half of the cruciform DNA. Panel D, gel retardation analysis of DNA binding reactions in which the complex (lanes 2, 6, 10; 1.5nM, lanes 3, 7, 11; 3nM) was incubated with 0.1nM of the cruciform DNA (lanes 2-4) or with 0.1nM of the duplex molecules (lanes 6-8, and 10-12).

Methods. Preparation of probes for length dependence experiment. Oligonucleotides containing bases -129 to -158 of the *ADH2* promoter were annealed to produce the 30bp probe. The 80bp probe was generated by PCR amplification of the -89 to -168 sequence of the *ADH2* promoter. Digestion of plasmid pHH26 with *Sal*I or *Eco*RI/*Hind*III released fragments of 134bp and 179bp respectively, that contained the -129 to -158 sequence.

FIGURE 4. SWI/SNF introduces positive supercoils into relaxed plasmid DNA in the presence of bacterial Topoisomerase I.

Panel A. SWI/SNF induced supercoiling. Closed relaxed plasmid DNA (lane 1) was incubated with the SWI/SNF complex and bacterial Topoisomerase I (3 units). Molar ratios of SWI/SNF to plasmid are indicated above each set of lanes. A ratio of 20:1 is 3nM SWI/SNF. Addition of ATP to a subset of the reactions is indicated. **Panel B.** SWI/SNF introduces positive supercoils. Plasmid DNA was supercoiled with SWI/SNF (3nM) and bacterial Topo I as in panel A. DNA was purified (lane 6) and retreated with either bacterial Topo I (lane 7), calf thymus Topo I (lane 8), or calf thymus Topo II (lane 9). Only the eukaryotic topoisomerases can relax the SWI/SNF-induced supercoils, indicating that they are positive supercoils. Lanes 1-4 show control reactions in which negatively supercoiled plasmid DNA was incubated with each topoisomerase in reaction conditions identical to lanes 7-9. **Panel C. A**

1 0

eukaryotic topoisomerase I blocks the ability of SWI/SNF to introduce positive supercoils. SWI/SNF complex (3nM) was incubated with relaxed plasmid DNA (lanes 1, 6) and either bacterial Topo I (lane 2, 2 units; lane 3, 20 units) or calf thymus Topo I (lane 4, 0.3 units; lane 5, 3 units).

Methods.   Supercoiling reactions (20 µl) contained 20 mM HEPES pH7.5/7 mM $MgCl_2$/15 mM KCl/0.5 mM DTT/50 µg per ml BSA/50 ng pJH28.   Reaction were incubated for 45 minutes at 30°C, stopped with 80 ul of STOP (1% SDS/10 mM EDTA/100 µg per ml proteinase K/ 50 µg per ml tRNA and incubated for 30 minutes at 37°C.   Samples were extracted with phenol/chloroform, ethanol precipitated, and samples were analyzed on 0.8% agarose gels and Southern analysis.   Blots were probed with pJH28 labeled with α^{32}P-dCTP by random priming.   Plasmid pJH28 contains *SUC2* sequences from -1100 to +14 in plasmid pRS316.

1 1

ADH2-1 -158AAATAGAGTGCCAGTAGCGACTTTTTTCAC-129

ADH2-2 $+77$TTCCAAAGCCAAAGCCCAACG$+97$

CYC1-a -162ACGACACATGATCATATGGCATGCATGTGCTCTGTATGTATAT-119

CYC1-b -221TAGCGTGGATGGCCAGGCAACTTT-198

TABLE 1. Sequence comparison of SWI/SNF binding sites.

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1 5

Design of a Potent Geranylgeranyltransferase I Inhibitor:

Disruption of Oncogenic K-Ras4B Processing and Signaling

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Running Title: GGTase I Inhibitor Disrupts Oncogenic K-Ras4B Processing and Signaling

The abbreviations used are: FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; PAGE, polyacrylamide gel electrophoresis; MAPK, mitogen activated protein kinase; CAAX, tetrapeptide where C = cysteine, A = aliphatic amino acid, and X = serine or methionine; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase inhibitor; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride

Abstract

Prenylation of the carboxyl terminal CAAX (C=cysteine, A=aliphatic and X=any amino acid) of Ras is required for its biological activity. We have designed a CAAX peptidomimetic, GGTI-287, which is 10 times more potent towards inhibiting geranylgeranyltransferase I (GGTase I) *in vitro* (IC_{50}=5 nM) than our previously reported farnesyltransferase (FTase) inhibitor, FTI-276. In whole cells, the methyl ester derivative of GGTI-287, GGTI-286, was 25-fold more potent (IC_{50}=2 μM) than the corresponding methyl ester of FTI-276, FTI-277, towards inhibiting the processing of the geranylgeranylated protein Rap1A. Furthermore, GGTI-286 is highly selective for geranylgeranylation over farnesylation since it inhibited the processing of farnesylated H-Ras only at much higher concentrations (IC_{50}>30 μM). While the processing of H-Ras was very sensitive to inhibition by FTI-277 (IC_{50}=100 nM), that of K-Ras4B was highly resistant (IC_{50}=10 μM). In contrast, we found the processing of K-Ras4B to be much more sensitive to GGTI-286 (IC_{50}=2 μM). Furthermore, oncogenic K-Ras4B stimulation of MAP Kinase was inhibited potently by GGTI-286 (IC_{50}=1 μM) but weakly by FTI-277 (IC_{50}=30 μM). Significant inhibition of oncogenic K-Ras4B stimulation of MAP kinase by GGTI-286 occurred at concentrations (1-3 μM) that did not inhibit oncogenic H-Ras stimulation of MAP kinase. The data presented in this manuscript provide the first demonstration of selective disruption of oncogenic K-Ras4B processing and signaling by a CAAX peptidomimetic. The higher sensitivity of K-Ras4B towards a GGTase I inhibitor has a tremendous impact on future research directions targeting Ras in anticancer therapy.

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Introduction

Ras is a small guanine nucleotide binding protein that cycles between its active (GTP-bound) and inactive (GDP-bound) forms to transduce growth and differentiation signals from receptor tyrosine kinases to the nucleus (1,2). Binding of epidermal and platelet-derived growth factors to their receptor tyrosine kinases results in autophosphorylation and recruitment of key signaling proteins to the receptor. Among these proteins are the Ras exchange factors that activate Ras by catalyzing the exchange of GDP for GTP. GTP-bound Ras activates a cascade of mitogen-activated protein (MAP) kinases by recruiting Raf to the plasma membrane. Raf, a serine/threonine kinase, phosphorylates MEK, which in turn, activates MAP kinase by phosphorylating it on threonine and tyrosine. Hyperphosphorylated MAP kinase translocates to the nucleus where it phosphorylates transcription factors that are involved in the regulation of growth-related genes. The growth signal is terminated when Ras hydrolyzes GTP to GDP (1-3). However, mutations that lock Ras in its GTP-bound form result in an uninterrupted growth signal and are believed to contribute to the development of more than one third of human cancers (4,5).

In order for Ras to transduce its normal and oncogenic signal it must be anchored to the plasma membrane, which is accomplished by posttranslational modifications that increase its hydrophobicity (6-8). A key step in this process is catalyzed by farnesyltransferase (FTase), an enzyme that transfers farnesyl from farnesylpyrophosphate, a cholesterol biosynthesis intermediate, to the cysteine of the carboxyl terminal CAAX of Ras (C=cysteine, A=aliphatic amino acid and X=serine or threonine) (9,10). A closely related enzyme, geranylgeranyltransferase I (GGTase I), attaches the lipid geranylgeranyl to the cysteine of the CAAX box of proteins where X is leucine (11,12). FTase and GGTase I are α/β heterodimers that share the α subunit (13,14). Cross-linking experiments suggested that both substrates (FPP and Ras CAAX) interact with the β subunit of FTase (15,16). Although GGTase I prefers leucine at the X position, its substrate specificity was shown to overlap with that of

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FTase *in vitro* (17). Furthermore, GGTase I was also able to transfer farnesyl to a leucine terminating peptide (18).

Because farnesylation of Ras is required for its oncogenic activity, we (19-22) and others (23-27) have designed potent inhibitors of FTase as potential anticancer drugs. These inhibitors are CAAX peptidomimetics which show great selectivity for FTase over GGTase I *in vitro* and selectively block the processing of farnesylated but not geranylgeranylated proteins in whole cells (22). Furthermore, FTase inhibitors can selectively block oncogenic Ras signaling and reverse malignant phenotype at concentrations that do not affect normal cells (24,25). However, mammalian cells express four types of Ras proteins (H-, N-, KA- and KB-Ras) among which K-Ras4B is the most frequently mutated form of Ras in human cancers (4,5). Although several laboratories have demonstrated potent inhibition of oncogenic H-Ras processing and signaling (26,28), this disruption has not been shown with K-Ras4B. Hence, a drawback of the previous studies is the use of H-Ras and not K-Ras4B as a target for the development of these inhibitors. Recently, we have shown that a potent inhibitor of FTase disrupts K-Ras4B processing but only at very high concentrations that also inhibited the processing of geranylgeranylated proteins (29). This suggested that K-Ras4B may be geranylgeranylated, particularly in cells where FTase is inhibited. Consistent with this possibility is the recent observation that K-Ras4B can be geranylgeranylated *in vitro*, but its K_m for GGTase I is 7 times higher than its K_m for FTase (30). GGTase I CAAX-based inhibitors that can block geranylgeranylation processing have not been reported. In the present study, we have designed a CAAX peptidomimetic that selectively inhibits GGTase I and demonstrate that oncogenic K-Ras4B processing and signaling is disrupted at concentrations that affect geranylgeranylation but not farnesylation processing.

Experimental Procedures

Synthesis of FTase and GGTase I Inhibitors - Peptidomimetics FTI-276 and FTI-277 were prepared as previously described (29). The GGTase I inhibitors GGTI-287 and 286 were prepared from 2-phenyl-4-nitrobenzoic acid (29) by reaction with L-leucine methyl ester followed by reduction with stannous chloride. The resulting 4-amino-2-phenylbenzoyl leucine methyl ester was reacted with N-Boc-S-trityl-cysteinal and deprotected by procedures similar to those described for the FTase inhibitors (29) to give GGTI-286 and -287 as their hydrochloride salts.

FTase and GGTase I Activity Assay - FTase and GGTase I activities from 60,000 x g supernatants of human Burkitt lymphoma (Daudi) cells (ATCC, Rockville, MD) were assayed exactly as described previously for FTase (22). Inhibition studies were performed by determining the ability of Ras CAAX peptidomimetics to inhibit the transfer of [³H]-farnesyl and [³H]-geranylgeranyl from [³H]FPP and [³H]GGPP to H-ras-CVLS and H-Ras-CVLL, respectively (22).

Ras and Rap1A Processing Assay - H-Ras cells (31) and K-Ras4B cells (32) were kind gifts from Dr. Channing Der and Dr. Adrienne Cox (University of North Carolina, Chapel Hill). Cells were seeded on day 0 in 100 mm dishes in Dulbecco's modified Eagles medium supplemented with 10% calf serum and 1% pen/strep. On days 1 and 2, cells were refed with medium containing various concentrations of FTI-277, GGTI-286 or vehicle (10 mM DTT in DMSO). On day 3, cells were washed and lysed in lysis buffer containing 50 mM HEPES, pH 7.5, 10 mM NaCl, 1% TX-100, 10% glycerol, 5 mM $MgCl_2$, 1 mM EGTA, 25 ug/ml leupeptin, 2 mM PMSF, 2 mM Na_3VO_4, 1 mg/ml soybean trypsin inhibitor, 10 ug/ml aprotinin, 6.4 mg/ml Sigma-104® phosphatase substrate. Lysates were cleared (14,000 rpm, 4°C, 15 min) and equal amounts of protein were separated on a 12.5% SDS-PAGE, transferred to

nitrocellulose, and immunoblotted using an anti-Ras antibody (Y13-259, ATCC) or an anti-Rap1A antibody (SC-65, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody reactions were visualized using either peroxidase-conjugated goat anti-rat IgG (for Y13-259), or peroxidase-conjugated goat anti-rabbit IgG (for Rap1A) and an enhanced chemiluminescence detection system (ECL, Amersham Corp.), as described previously (22).

MAP Kinase Immunoblotting - Cells were treated with FTI-277, GGTI-286, or vehicle and lysed as previously described for Ras and Rap1A processing. Equal amounts of protein were separated on a 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-MAP kinase antibody (erk2, monoclonal, UBI, Lake Placid, NY). Antibody reactions were visualized using peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and an enhanced chemiluminescence detection system (ECL, Amersham Corp.).

Results and Discussion

The carboxyl terminal CAAX tetrapeptide of Ras is a substrate for FTase and serves as a target for designing inhibitors of this enzyme with potential anticancer activity (23). We have recently made a highly potent ($IC_{50}=500$ pM) inhibitor of FTase, FTI-276 (Fig. 1) (29). Its cell-permeable methylester FTI-277 inhibited H-Ras processing in whole cells with an IC_{50} of 100 nM (29). Furthermore, FTI-276 is highly selective (100-fold) for FTase over GGTase I (Table 1). Although oncogenic H-Ras processing and signaling were exquisitely sensitive to FTI-277, those of K-Ras4B were highly resistant. However, at high concentrations of FTI-277, when the processing of the geranylgeranylated Rap1A protein was inhibited, K-Ras4B processing was also inhibited (29). We, therefore, set out to determine whether a GGTase I-selective inhibitor would disrupt K-Ras4B processing and signaling. Our approach involved

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replacing the central "AA" of CAAX tetrapeptides by a hydrophobic spacer and incorporating a leucine

residue in the C-terminal position to optimize recognition by GGTase I. We herein report a CAAL

peptidomimetic, GGTI-287, where reduced cysteine is linked to leucine by 2-phenyl-4-amino benzoic acid

(Fig. 1). The phenyl substituent was designed to occupy the hydrophobic "AA" binding pocket that must

be present in the enzyme. GGTI-287 potently inhibited GGTase I *in vitro* (IC_{50} = 5 nM) and was selective

towards inhibiting GGTase I over FTase (IC_{50}=25 nM) (Table 1). Thus, the substitution of methionine

in FTI-276 by a leucine in GGTI-287 (Fig. 1) increased the potency towards GGTase I by approximately

10-fold (Table 1). More importantly, it reversed the selectivity from a FTase to a GGTase I-specific

inhibitor by a factor of 500 (Table 1). To determine whether this selectivity is respected in whole cells,

we have synthesized the cell-permeable methyl ester derivative of GGTI-287, GGTI-286 (Fig. 1), and

treated NIH 3T3 cells which overexpress oncogenic H-Ras-CVLS (31) with GGTI-286 (0-30 μM). Cell

lysates were electrophoresed on SDS-PAGE and immunoblotted with an anti-Ras antibody as described

under "Experimental Procedures". Figure 2 shows that accumulation of unprocessed H-Ras did not occur

until 30 μM GGTI-286. Therefore, GGTI-286 is not a good inhibitor of H-Ras processing in whole cells.

However, GGTI-286 was a very potent inhibitor of the processing of the geranylgeranylated Rap1A

protein (IC_{50}=2 μM) (Fig. 2). Thus, GGTI-286 is more than 15-fold selective for inhibition of

geranylgeranylation over farnesylation processing (Table 1). This data is in direct contrast to the FTase

specific inhibitor FTI-277 which inhibited H-Ras and Rap1A processing with IC_{50}s of 100 nM and 50

μM, respectively (Fig. 2). Thus, GGTI-286 is 25-fold more potent than FTI-277 at inhibiting

geranylgeranylation in whole cells (Table 1).

We next evaluated the ability of GGTI-286 to inhibit the processing and signaling of oncogenic

K-Ras4B. NIH 3T3 cells which overexpress oncogenic K-Ras4B (32) were treated with either GGTI-286

(0-30 μM) or FTI-277 (0-30 μM) and the lysates were immunoblotted with an anti-Ras antibody as

described under "Experimental Procedures". Figure 3 shows that GGTI-286 inhibited potently the

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processing of K-Ras4B with an IC_{50} of 2 μM. The ability of GGTI-286 to inhibit the processing of K-Ras4B was much closer to its ability to inhibit the processing of geranylgeranylated Rap1A (IC_{50}=2 μM) than that of farnesylated H-Ras (IC_{50}>30 μM) (Fig. 2) (Table 1). This suggested that K-Ras4B might be geranylgeranylated. Consistent with this is the fact that K-Ras4B processing was very resistant to the FTase-specific inhibitor FTI-277 (IC_{50}=10 μM) (Fig. 3). Furthermore, GGTI-286 inhibited K-Ras4B processing at concentrations (1-3 μM) (Fig. 3) that had no effect on the processing of farnesylated H-Ras (Fig. 2). These results are not consistent with the work of Casey et al (7) who used [^{3}H] mevalonic acid to label cellular proteins and provided evidence for a farnesylated K-Ras4B based on HPLC of the radiolabelled prenyl group. However, mass of the prenyl group in these studies was not determined and this may have led to an erronious conclusion.

To determine whether inhibition of K-Ras4B processing by GGTI-286 results in disruption of oncogenic signaling, we evaluated the ability of GGTI-286 to antagonize oncogenic K-Ras 4B constitutive activation of MAP kinase. Activated MAP kinase is hyperphosphorylated and migrates slower than hypophosphorylated (inactive) MAP kinase on SDS-PAGE (26,29). Figure 4 shows that K-Ras4B transformed cells contained mainly activated MAP kinase. Treatment of these cells with the FTase-specific inhibitor FTI-277 (0-30 μM) did not inhibit MAP kinase activation until 30 μM (Fig. 4). In contrast, GGTI-286 inhibited MAP kinase activation with an IC_{50} of 1 μM and the block was complete at 10 μM. Thus, GGTI-286 blocked oncogenic K-Ras4B MAP kinase activation at a concentration (10 μM) where FTI-277 had no effect. In contrast, oncogenic H-Ras activation of MAP kinase was inhibited only slightly by GGTI-286 whereas FTI-277 completely blocked this activation at 3 μM (Fig. 4). Furthermore, GGTI-286 blocked K-Ras4B activation of MAP kinase at a concentration (10 μM) that had little effect on H-Ras activation of MAP kinase (Fig. 4). It should be noted that GGTI-286 was not toxic to cells at concentrations as high as 10 μM. However, at higher concentrations (30 μM), GGTI-286 did

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show some signs of toxicity as reflected by a rounded morphology of the cells. Thus, GGTI-286 was not toxic at concentrations (10 μM) that resulted in complete inhibition of MAP kinase activation.

Recently, we have demonstrated that the FTase-specific inhibitor FTI-277 inhibits oncogenic H-Ras processing and signaling (29) and blocks in vivo tumor growth of H-Ras transformed NIH 3T3 cells and a human lung carcinoma that expresses a K-RasA mutation (33). However, processing of K-Ras4B was inhibited by FTI-277 only at high concentrations similar to those needed to inhibit the processing of the geranylgeranylated protein Rap1A (29). In the present study, we have described the design of a geranylgeranylation-specific inhibitor and its effects on oncogenic K-Ras4B processing and signaling. Our results demonstrate that oncogenic K-Ras4B processing and constitutive activation of MAP kinase are potently inhibited by a GGTase-I-selective inhibitor (GGTI-286) but are resistant to one selective for FTase (FTI-277). This is in direct contrast to the processing and signaling of oncogenic H-Ras which was very sensitive to FTI-277 and highly resistant to GGTI-286. The resistance of K-Ras4B to disruption by FTase inhibitors could be explained by the 50-fold higher affinity of K-Ras4B for FTase compared to H-Ras (30). Our current data strongly suggest, however, that K-Ras4B may be resistant to FTase inhibition because it is posttranslationally processed by a geranylgeranyl rather than a farnesyl group. This is consistent with the recent observation that in vitro K-Ras4B can be geranylgeranylated by GGTase I (30). Although this previous work shows that K-Ras4B is a 7 times better substrate in vitro for FTase (K_m=0.2 μM) than GGTase-I (K_m=1.5 μM) (30), our data suggest that, in cultured cells, K-Ras4B is geranylgeranylated. This is supported by the fact that GGTI-286 inhibited oncogenic K-Ras4B processing and MAP kinase activation at concentrations (1 and 3 μM) that did not affect farnesylation-dependent processing.

The results presented in this study are critical to the further design and development of inhibitors of Ras prenylation as potential anticancer agents. The results identify the GGTase I-specific inhibitor GGTI-286 as a small molecule capable of antagonizing selectively oncogenic K-Ras4B (not H-Ras)

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signaling. This is a key finding since K-Ras4B is the most frequently identified mutated Ras in human cancers and its function has been resistant to FTase inhibitors. Finally, the availability of K-Ras4B-selective inhibitors (i.e., GGTI-286) in addition to H-Ras-selective inhibitors (i.e. FTI-277) will enhance our understanding of the distinctive roles of these two forms of Ras in normal and oncogenic signaling.

Figure 1.   **CAAX peptidomimetic structures.**

Structures of FTI-276/277 and GGTI-287/286.

Figure 2.   **Disruption of H-Ras and Rap1A processing.**

NIH 3T3 cells that overexpress oncogenic H-Ras were treated with various concentrations of FTI-277 (0-50 μM) or GGTI-286 (0-30 μM). The cells were lysed and the lysates were electrophoresed on SDS-PAGE and immunoblotted with either anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures". U and P designate unprocessed and processed forms of the proteins. Data are representative of three independent experiments.

Figure 3. Disruption of K-Ras4B processing.

NIH 3T3 cells that overexpress oncogenic K-Ras4B were treated with FTI-277 or GGTI-286 (0-30 μM). The cells were lysed and the lysates were electrophoresed on SDS-PAGE and immunoblotted with anti-Ras antibodies as described under "Experimental Procedures". U and P designate unprocessed and processed forms of Ras. The data are representative of three independent experiments.

Figure 4.   **Inhibition of oncogenic activation of MAP Kinase.**

NIH 3T3 cells that overexpress either oncogenic H-Ras or K-Ras4B were treated with either FTI-277 or GGTI-286 (0-30 μM). The cells were lysed and the lysates were electrophoresed on SDS-PAGE and immunoblotted with an anti-MAP kinase antibody. P-MAPK designates hyperphosphorylated MAP kinase. The data are representative of three independent experiments.

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TABLE 1

In Vitro (IC$_{50}$, nM)

	FTase	GGTase I
FTI-276	0.5	50
GGTI-287	25	5

In Vivo Processing (IC$_{50}$, uM)

	H-Ras	K-Ras	Rap1A
FTI-277	0.1	10	50
GGTI-286	>30	2	2

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Fig. 2

Fig. 3

K-Ras4B

FTI-277   GGTI-286

0 1 3 10 30   1 3 10 30

Ras U
    P

Fig. 4

K-Ras4B

FTI-277   GGTI-286

0 1 3 10 30 1 3 10 30

P-MAPK →

H-Ras

FTI-277   GGTI-286

0 0.1 0.3 1 3 10 1 3 10 30

P-MAPK →

Communication

Ras CAAX Peptidomimetic FTI-277 Selectively Blocks Oncogenic Ras Signaling by Inducing Cytoplasmic Accumulation of Inactive Ras-Raf Complexes*

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Ras-induced malignant transformation requires Ras farnesylation, a lipid posttranslational modification catalyzed by farnesyltransferase (FTase). Inhibitors of this enzyme have been shown to block Ras-dependent transformation, but the mechanism by which this occurs remains largely unknown. We have designed FTI-276, a peptide mimetic of the COOH-terminal Cys-Val-Ile-Met of K-Ras4B that inhibited potently FTase in vitro (IC$_{50}$ = 500 pM) and was highly selective for FTase over geranylgeranyltransferase I (GGTase I) (IC$_{50}$ = 50 nM). FTI-277, the methyl ester derivative of FTI-276, was extremely potent (IC$_{50}$ = 100 nM) at inhibiting H-Ras, but not the geranylgeranylated Rap1A processing in whole cells. Treatment of H-Ras oncogene-transformed NIH 3T3 cells with FTI-277 blocked recruitment to the plasma membrane and subsequent activation of the serine/threonine kinase c-Raf-1 in cells transformed by farnesylated (H-RasF), but not geranylgeranylated, Ras (H-RasGG). FTI-277 induced accumulation of cytoplasmic non-farnesylated H-Ras that was able to bind Raf and form cytoplasmic Ras/Raf complexes in which Raf kinase was not activated. Furthermore, FTI-277 blocked constitutive activation of mitogen-activated protein kinase (MAPK) in H-RasF, but not H-RasGG, or Raf-transformed cells. FTI-277 also inhibited oncogenic K-Ras4B processing and constitutive activation of MAPK, but the concentrations required were 100-fold higher than those needed for H-Ras inhibition. The results demonstrate that FTI-277 blocks Ras oncogenic signaling by

accumulating inactive Ras/Raf complexes in the cytoplasm, hence preventing constitutive activation of the MAPK cascade.

Ras proteins are plasma membrane-associated GTPases that function as relay switches transducing biological information from extracellular signals to the nucleus (for review, see Refs. 1–3). In normal cells, Ras proteins cycle between the GDP (inactive)- and GTP (active)-bound forms to regulate proliferation and differentiation. The mechanism by which extracellular signals, such as epidermal and platelet-derived growth factor, transduce their biological information to the nucleus via Ras proteins has recently been unraveled. Binding of these growth factors to tyrosine kinase receptors results in autophosphorylation of various tyrosines which bind src-homology 2 (SH2) domains of several signaling proteins. One of these, a cytoplasmic complex of GRB-2 and a Ras exchanger (SOS-1), is recruited by the tyrosine-phosphorylated receptor, where SOS-1 catalyzes the exchange of GDP for GTP on Ras, hence activating it. GTP-bound Ras recruits c-Raf-1, a serine/threonine kinase, to the plasma membrane where its kinase activity is activated by as yet undetermined membrane-associated events. Raf triggers a kinase cascade by phosphorylating MAP kinase kinase (MEK) which, in turn, phosphorylates MAPK¹ on threonine and tyrosine residues. Activated MAPK translocates to the nucleus where it phosphorylates transcription factors. In a large number of human cancers, Ras is locked in its GTP-bound form due to mutations in amino acids 12, 13, or 61 (4, 5). As a result, the Ras pathway no longer requires an upstream growth signal, is uninterrupted and the enzymes in this pathway such as Raf, MEK, and MAPK are constitutively activated (1–3).

In addition to its inability to hydrolyze GTP, oncogenic Ras must associate with the plasma membrane to cause malignant transformation (6–8). Ras membrane association is mediated through a series of posttranslational modifications (9–12). The first step is catalyzed by a cytosolic heterodimer farnesyltransferase (FTase), which attaches farnesyl to the thiol group of cysteine of the carboxyl-terminal tetrapeptide CAAX, where A is isoleucine or valine and X is serine or methionine (13–16). Because farnesylation is required and sufficient for Ras transformation (8, 17), FTase is an attractive target for the development of a potential new class of anti-cancer agents (18, 19). Although CAAX peptides are potent competitive inhibitors of FTase, rapid degradation and low cellular uptake limit their use as therapeutic agents. Over the last 3 years, we (20–22) and others (23–26) have designed CAAX peptidomimetics that potently inhibit FTase in vitro and Ras processing in vivo but that retain several peptidic features. More recently, we have designed non-peptide CAAX mimetics that have several desirable features for further development as anti-cancer agents (27). Although these non-peptide mimics and CAAX peptidomimetics inhibit FTase potently (nM), their ability to disrupt Ras

¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; CAAX, tetrapeptide where C = cysteine, A = aliphatic amino acid, and X = serine or methionine; FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; FTI, farnesyl transferase inhibitor; Boc, t-butoxycarbonyl; HPLC, high performance liquid chromatography.

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processing in whole cells occurs at concentrations (100 μM) that would not be easily achievable in *in vivo* settings. Therefore, there is a need for improved FTase inhibitors with more potent activity in whole cells and *in vivo*.

Ras *CAAX* peptidomimetics have been shown to reverse oncogenic H-Ras transformation, inhibit the growth of H-Ras-transformed, but not normal cells in culture, and slow the growth of Ras but not Raf-transformed cells in nude mice (23, 24, 28). Recently, FTase inhibitors have also been shown to inhibit oncogenic Ras activation of MAPK in H-Ras-transformed cells (29, 30). Whether FTase inhibitors also inhibit oncogenic K-Ras signaling is not yet known. This is an important question, since K-Ras is a more efficient substrate for FTase, rendering it more difficult to block by FTase inhibitors, and since K-Ras mutations are more prevalent in human tumors where Ras is mutated. Furthermore, the mechanism by which FTase inhibitors suppress MAPK activation has not been investigated. Specifically, the effects of FTase inhibitors on the interactions between Ras and its downstream effectors such as Raf have not been studied. The present work describes the design of a highly potent (pM/nM) Ras *CAAX* peptidomimetic which antagonizes both H- and K-Ras oncogenic signaling. The results demonstrate that the mechanism by which this inhibitor blocks Ras-dependent signaling involves sequestering Raf in the cytoplasm away from the plasma membrane where it would be activated.

EXPERIMENTAL PROCEDURES

Synthesis of CAAX Analogues—The peptidomimetic FTI-276 was synthesized as follows: 2-bromo-4-nitrotoluene was coupled with phenylboronic acid. The coupled product was oxidized with $KMnO_4$ to 2-phenyl-4-nitrobenzoic acid which was coupled with L-methionine methyl ester followed by reduction with stannous chloride. The resulting 4-amino-2-phenylbenzoyl methionine methyl ester was reacted with *N*-Boc-*S*-trityl-cysteinal to give *N*-Boc-*S*-trityl methyl ester of FTI-276. This methyl ester was hydrolyzed by LiOH and then deprotected by trifluoroacetic acid. The pure FTI-276 was obtained through preparative HPLC. The peptidomimetic FTI-277 was made from the *N*-Boc-*S*-trityl methyl ester of FTI-276 by first treatment with mercuric chloride followed by hydrogen sulfide in methanol. The final product, FTI-277, was obtained as its hydrochloride salt. Spectroscopic data of both FTI-276 and FTI-277 were consistent with their assigned structures. HPLC analysis showed purity over 99.9%.

FTase and GGTase I Activity Assays—FTase and GGTase I activities from 60,000 × *g* supernatants of human Burkitt lymphoma (Daudi) cells (ATCC, Rockville, MD) were assayed exactly as described previously (27). Inhibition studies were performed by determining the ability of Ras *CAAX* peptidomimetics to inhibit the transfer of [^{3}H]farnesyl and [^{3}H]geranylgeranyl from [^{3}H]farnesyl pyrophosphate and [^{3}H]geranylgeranyl pyrophosphate to H-Ras-CVLS and H-Ras-CVLL, respectively (27).

Ras and Rap1A Processing Assay—H-RasF, H-RasGG, pZIPneo, Raf, S186 (31), and K-Ras4B cells (17) were seeded on day 0 in 100-mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% penicillin/streptomycin. On days 1 and 2, cells were refed with medium containing various concentrations of FTI-277 or vehicle (10 mM DTT in Me$_2$SO). On day 3, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 25 μg/ml leupeptin, 1 mM PMSF, 2 mM Na$_3$VO$_4$, 1 mg/ml soybean trypsin inhibitor, and 6.4 mg/ml Sigma-104® phosphatase substrate. Lysates were then immunoblotted using an anti-Ras antibody (Y13-238, ATCC) or an anti-Rap1A antibody (SC-65, Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (27).

Co-immunoprecipitation of Raf and Ras—Cells were seeded on day 0 in 100-mm dishes, and on days 1 and 2 cells were treated with FTI-277 (10 μM) or vehicle. On day 3, cells were collected and pellets were resuspended in ice-cold hypotonic buffer (10 mM Tris, pH 7.5; 5 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF) and sonicated to break up the cell pellet. The cell suspension was then centrifuged at 2,000 rpm for 10 min to clear debris after which the supernatant was spun for 30 min at 100,000 × *g* to separate membrane and cytosol fractions. The cytosol and membrane fractions were then lysed as described previously (32).

Equal amounts of cytosol and membrane fractions were immunoprecipitated using 50 μl of a 25% protein A-Sepharose CL-4B suspension (Sigma) with 1 μg/ml anti-c-Raf-1 (SC133, Santa Cruz Biotechnology, Santa Cruz, CA). The samples were washed five times in 50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl$_2$, 0.1% Triton X-100, 10% glycerol, 20 mM NaF). The final pellets were run on 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the presence of Ras using anti-Ras antibody (Y13-238) and anti-Raf antibody (SC133, Santa Cruz Biotechnology, Santa Cruz, CA). Detection was the same as above for Ras and Rap1A processing.

Detection of GTP and GDP Bound to Ras—H-RasF cells were seeded and treated as above for Ras/Raf interaction studies. On day 2, however, cells were labeled overnight with [^{32}P]orthophosphate as described previously (32). On day 3, the cytosol and membrane fractions were separated, lysed, and equal amounts of lysate were immunoprecipitated using anti-Ras antibody (Y13-259) along with 30 μl of protein A-agarose goat anti-rat IgG complex (Oncogene Science). Bound nucleotide was eluted and separated by TLC as described previously (32).

Raf-1 Kinase Activity Assay—Raf-1 kinase activity was assayed by determining the ability of Raf to transfer phosphate from [γ-^{32}P]ATP to a 19-mer peptide containing a Raf autophosphorylation site. Membrane and cytosol fraction isolation and Raf immunoprecipitation were described above. Immunoprecipitates were washed with kinase buffer (50 mM Tris, pH 7.3, 150 mM NaCl, 12 mM MnCl$_2$, 1 mM DTT, 0.2% Tween 20). Immune complex kinase assays were performed by incubating immunoprecipitates from membrane and cytosol fractions in 96 μl of kinase buffer with 20 μCi of [γ-^{32}P]ATP (10 mCi/ml, Amersham Corp.) and 2 μl of the Raf-1 substrate peptide IVQQFGFQRRASDDGKLTD (1 mg/ml, Promega, Madison, WI) for 30 min at 25 °C. The phosphorylation reaction was terminated by spotting a 50-μl aliquot onto Whatman P81 phosphocellulose filters. The filters were washed in 0.5% orthophosphoric acid and air-dried. The amount of ^{32}P incorporated was determined by liquid scintillation counting.

MAP Kinase Activity Assay—Cells were treated with FTI-277 and lysed as described above for Ras and Rap1A processing. Equal amounts of lysate were separated on a 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-MAPK antibody (erk2, monoclonal, Upstate Biotechnology, Inc., Lake Placid, NY). Antibody reactions were visualized using peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence detection (Amersham).

RESULTS AND DISCUSSION

Recently, we (20–22, 27) and others (23–26) have designed Ras *CAAX* peptidomimetics that inhibit FTase potently with concentrations in the nM range. However, these agents inhibited Ras processing in whole cells only at μM levels (29, 30). In order to investigate the mechanism of action of FTase inhibitors, we sought to first improve the potency and selectivity of our first generation of *CAAX* peptidomimetics. Structure activity relationship studies with *CAAX* peptides and peptidomimetics predict a hydrophobic region in the active site of FTase that interacts with the central portion of the *CAAX* tetrapeptide. In our original designs (20–22), we have replaced the central aliphatic dipeptide "VI" in CVIM by aromatic spacers of the aminobenzoic acid family (Fig. 1A). Structural comparison of CVIM with the peptidomimetic FTI-249 (Fig. 1A) suggests that increased binding energy could be gained by increasing the size and hydrophobicity of the aminobenzoic acid spacer to fully occupy the FTase substrate binding pocket. In the present work, we have designed FTI-276 and its methyl ester FTI-277 (Fig. 1A), where reduced cysteine and methionine are linked by 2-phenyl-4-aminobenzoic acid, hence increasing the hydrophobic character of the central portion of the peptidomimetic. FTI-276 and FTI-277 were synthesized as described under "Experimental Procedures." Fig. 1B shows that FTI-276 inhibited FTase with an IC$_{50}$ of 500 pM, whereas FTI-249, the unsubstituted precursor to FTI-276, had an IC$_{50}$ of 200,000 pM (27). Thus, a phenyl ring at the 2 position of the aminobenzoic acid spacer increased inhibition potency of FTase by 400-fold, indicating a significant role for the hydrophobic pocket within the *CAAX* binding site of FTase. This extremely potent inhibitor was also highly selective (100-fold) for FTase over the closely related GGTase I. FTI-276 inhibited GGTase I with an IC$_{50}$ of
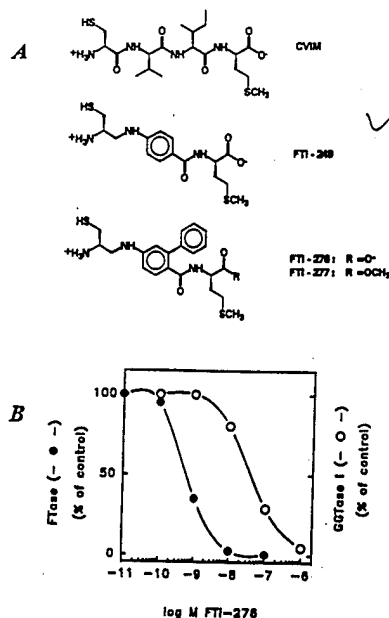
A

B

FIG. 1. Ras *CAAX* peptidomimetics and FTase/GGTase I inhibition. *A*, structures of CVIM, FTI-249, FTI-276, and FTI-277. *B*, FTase and GGTase I inhibition assays were carried out by determining the ability of FTI-276 to inhibit the transfer of farnesyl and geranylgeranyl to recombinant H-Ras-CVLS and H-Ras-CVLL, respectively. The data are representative of at least three independent experiments.

FIG. 2. Effects of FTI-277 on Ras and Rap1A processing. *A*, H-RasF cells were treated with various concentrations of FTI-277, lysed, and the lysates were immunoblotted with anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." *B*, pZIPneo, H-RasF, H-RasGG, Raf, and S186 cells were treated with vehicle or FTI-277 (10 μM), lysed, and lysates were immunoblotted by anti-Ras antibody. Data are representative of five independent experiments.

50 nM (Fig. 1*B*). This 100-fold selectivity is superior to our previously reported 15-fold selectivity of the parent compound FTI-249 (27). We next determined the ability of FTI-276 to inhibit Ras processing. To facilitate cellular uptake, we have used the corresponding methyl ester, FTI-277 (Fig. 1*A*). H-RasF cells (NIH 3T3 cells transformed with oncogenic (61 leucine) H-Ras-CVLS (31)) were treated with FTI-277 (0–50 μM), and the lysates were blotted with anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." Fig. 2*A* shows that concentrations as low as 10 nM inhibited Ras processing but concentrations as high as 10 μM did not inhibit processing of the geranylgeranylated Rap1A (Fig. 2*A*). FTI-277 inhibited Ras processing with an IC$_{50}$ of 100 nM (Fig. 2*A*), whereas the IC$_{50}$ of FTI-249 was 100 μM. Furthermore, the most potent *CAAX* peptidomimetics previously reported inhibited Ras processing in whole cells at concentrations of 10 μM or higher (28–30). The selectivity of FTI-277 for farnesylation over geranylgeranylation processing in whole cells was further investigated by treating H-RasGG cells (NIH 3T3 cells transformed with oncogenic (leucine 61) H-Ras-CVLL (31)) with FTI-277. Fig. 2*B* shows that the processing of H-RasGG was not affected, whereas that of H-RasF was completely blocked. Furthermore, the processing of endogenous Ras was also blocked in pZIPneo cells (NIH 3T3 cells transfected with empty vector) and Raf cells (NIH 3T3 cells transfected with a transforming mutant of human Raf-1 (Raf22W) (33)). Thus, FTI-277 is a farnesylation-specific inhibitor which blocks the processing of both oncogenic and normal Ras.

In order to determine the mechanism by which FTI-277 disrupts Ras oncogenic signaling, we transfected NIH 3T3 cells with activated (GTP-locked) Ras and first investigated the effects of FTI-277 on the interaction of Ras with its immediate
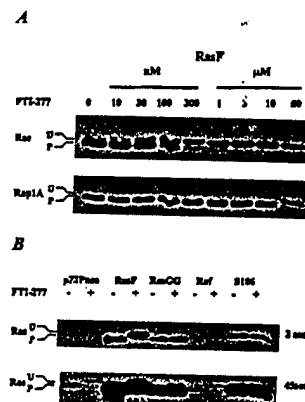
effector c-Raf-1 (1–3, 32). Various NIH 3T3 cell transfectants (pZIPneo, H-RasF, H-RasGG) were treated with vehicle or FTI-277, membrane and cytosolic fractions were isolated and immunoprecipitated with anti-Raf antibody, and the resulting immunoprecipitates were blotted with anti-Ras antibody as described under "Experimental Procedures." Fig. 3 shows that Raf did not associate with Ras in pZIPneo cells which do not contain GTP-locked Ras. In contrast, H-RasF and H-RasGG cells contain Ras-Raf complexes in the membrane but not in the cytosolic fractions of untreated cells (Fig. 3). Treatment with FTI-277 resulted in the accumulation of Ras-Raf complexes in the cytoplasmic but not membrane fractions of H-RasF cells (Fig. 3). The lack of Ras-Raf interaction at the cell membrane and accumulation of these complexes in the cytoplasm occurred only in Ras-F but not Ras-GG cells in agreement with the Ras processing selectivity results of Fig. 2. Thus, our results demonstrate that inhibition with FTI-277 results in the accumulation of non-farnesylated cytosolic Ras that is capable of binding to Raf. The fact that non-processed Ras can associate with Raf in a non-membranous, cytoplasmic environment was confirmed by transfecting NIH 3T3 cells with a GTP-locked Ras that lacks a farnesylation site (Ras mutant with a leucine 61 oncogenic mutation and a serine 186 mutation (34)) and, therefore, remains in the cytoplasm. These cells were shown to contain only cytoplasmic Ras-Raf complexes when immunoprecipitated with Raf and blotted with anti-Ras antibodies (Fig. 3, *S186*). Thus, farnesylation is not required for Ras to bind to Raf. Furthermore, the fact that non-farnesylated Ras binds Raf in the cytoplasm gives support to an earlier suggestion that unprocessed GTP-locked Ras is a dominant negative form of Ras (35).

Since Raf binds Ras-GTP with much higher affinity than Ras-GDP (1–3), we determined the nucleotide state of Ras in the cytoplasmic Ras-Raf complexes as described under "Experimental Procedures." Fig. 4*A* shows that in H-RasF cells only membrane fractions contained GTP-locked Ras. Upon treatment with FTI-277, however, GTP-locked H-Ras was found primarily in the cytosol (Fig. 4*A*). Thus, the cytoplasmic form of H-Ras(61L) is still GTP-bound and can, therefore, still interact with Raf. We next determined the Ser/Thr kinase activity of Raf in Ras/Raf complexes by immunoprecipitating Raf and assaying for its ability to phosphorylate a 19-mer autophospho-

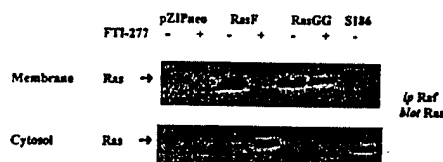4   *FTI-277 Induces Cytoplasmic Ras-Raf Complexes*

Fig. 3. Effects of FTI-277 on Ras/Raf association. pZIPneo, H-RasF, H-RasGG, and S186 cells were treated with vehicle or FTI-277 (10 μM), homogenized, and the membrane and cytosolic fractions were separated and immunoprecipitated by an anti-Raf antibody as described under "Experimental Procedures." The immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with anti-Ras antibody. Data are representative of three independent experiments.
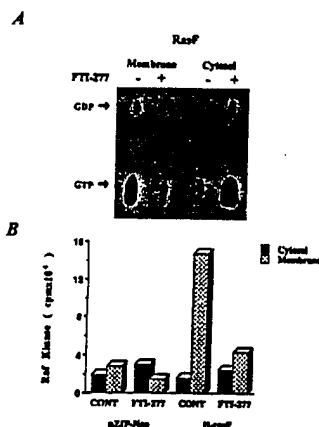

Fig. 4. Effects of FTI-277 on Ras nucleotide binding and Raf kinase activity. A, H-RasF cells were treated with vehicle or FTI-277, lysed, and the lysates were immunoprecipitated with anti-Ras antibody. GTP and GDP were then released from Ras and separated by TLC as described under "Experimental Procedures." B, pZIPneo and H-RasF cells were treated with vehicle or FTI-277, lysed, and cell lysates were immunoprecipitated with an anti-Raf antibody. Raf kinase was assayed as described under "Experimental Procedures." Data are representative of three independent experiments.

rylated peptide. Fig. 4B shows that oncogenic H-RasF induced activation of Raf at the plasma membrane and that treatment with FTI-277 suppressed this activation. More importantly, the cytoplasmic Ras/Raf complexes (Fig. 3) had basal levels of Raf kinase activity that were comparable with those of the parental NIH 3T3 cell line pZIPneo (Fig. 4B). Taken together, Figs. 3 and 4 demonstrate that oncogenic transformation with GTP-locked H-Ras results in the constitutive recruitment of Raf to the plasma membrane and its subsequent activation. Furthermore, FTase inhibition by FTI-277 suppresses this activation by inducing the accumulation of Ras-Raf complexes in the cytoplasm where Ras is GTP-bound, but Raf kinase is not activated. The fact that Raf kinase is not activated when bound to Ras in a non-membranous environment is consistent with recent reports that indicate that Raf activation requires an as yet unidentified activating factor at the plasma membrane (36).

We then investigated the effects of FTI-277 on oncogenic Ras activation of MAPK, a Raf downstream signaling event (1–3). Oncogenic activation of MAPK can be easily detected, since the phosphorylated activated MAPK migrates slower in SDS-PAGE (29). Fig. 5A shows that NIH 3T3 cells transfected with pZIPneo contain only inactive MAPK but that upon transformation with oncogenic H-Ras, MAPK is activated (Fig. 5A).
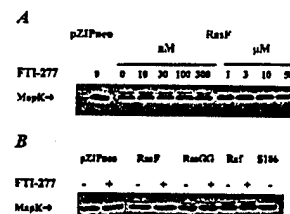

Fig. 5. Effect of FTI-277 on oncogenic activation of MAPK. A, H-RasF cells were treated with various concentrations of FTI-277, cells lysed, and lysates run on SDS-PAGE and immunoblotted with anti-MAPK antibody as described under "Experimental Procedures." B, pZIPneo, H-RasF, H-RasGG, Raf, and S186 cells were treated with vehicle or FTI-277 (10 μM), lysed, and cells lysates processed as for A. Data are representative of two independent experiments.

Pretreatment with FTI-277 results in a concentration-dependent inhibition of the constitutive activation of MAPK by oncogenic H-Ras. Concentrations as low as 300 nM were effective, and the inhibition was complete at 1 μM. Taken together, Figs. 2 and 5 demonstrate that at least 50% but less than 100% inhibition of H-Ras processing is required for inhibition of MAPK activation. To determine whether the inhibition of MAPK activation is due to selectively antagonizing H-Ras function we have used a series of NIH 3T3 cells lines transformed with various oncogenes. Fig. 5B shows that FTI-277 was able to block H-RasF but not H-RasGG activation of MAPK, and this is consistent with its ability to inhibit H-RasF but not H-RasGG processing. Selectivity of FTI-277 toward inhibition of Ras-dependent activation of MAPK was substantiated by using NIH 3T3 cells, where MAPK is constitutively activated by transformation with the Raf oncogene (33). Fig. 5B shows that oncogenic Raf activation of MAPK is not blocked by FTI-277, even though processing of endogenous Ras was inhibited in these cells (Fig. 2B). Taken together these results clearly demonstrate that FTI-277 is highly effective and selective in disrupting constitutive H-Ras-specific activation of MAPK.

Since K-Ras4B, the predominant form of Ras mutated in human tumors, is a much more efficient substrate (CAAX = CVIM) for FTase than is H-Ras (CAAX = CVLS) (13), its processing has been difficult to disrupt. To determine whether or not FTI-277 inhibits K-Ras processing, we have treated K-Ras4B cells (NIH 3T3 cells transformed with oncogenic (variant line 12) K-Ras4B-CVIM (17)) with FTI-277 (0–30 μM). Fig. 6 shows that FTI-277 inhibited K-Ras4B processing with an IC50 of 10 μM. Thus, inhibiting K-Ras4B processing (Fig. 6) requires 100-fold higher concentration than that needed for inhibition of H-Ras processing (Fig. 2A). This lower sensitivity to FTI-277 could be because K-Ras4B-CVIM is a much better substrate than H-Ras-CVLS (13). An alternative explanation is that K-Ras4B-CVIM could be geranylgeranylated (40), especially when cellular FTase is inhibited. The fact that inhibition of K-Ras4B processing occurs only at concentrations that inhibit the processing of the geranylgeranyiated Rap1A (Fig. 2A) is consistent with this latter possibility. We next determined whether the inhibition of K-Ras processing results in disruption of oncogenic K-Ras4B constitutive activation of MAPK. The same cell lysates that were blotted with anti-Ras antibody (Fig. 6) were reblotted with anti-MAPK antibody as described under "Experimental Procedures." Fig. 6 shows that NIH 3T3 cells that overexpress oncogenic K-Ras4B (17) contain mainly hyperphosphorylated (activated) MAPK. Treatment of these cells with FTI-277 (30 μM) inhibited oncogenic K-Ras4B constitutive activation of MAPK (Fig. 6). Furthermore, consistent with inhibition of Ras processing data (Fig. 6), higher concentrations were required to inhibit MAPK activation by K-Ras4B
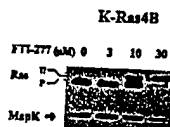
K-Ras4B

FIG. 6. **FTI-277 inhibits oncogenic K-Ras4B processing and activation of MAPK.** NIH 3T3 cells that overexpress oncogenic K-Ras4B were treated with FTI-277 (0, 3, 10, and 30 μM), and the cell lysates were immunoblotted with anti-Ras or anti-MAPK as described under "Experimental Procedures." Data are representative of three and two independent experiments, respectively.

as compared with H-Ras. Nevertheless, the data clearly demonstrate for the first time that an FTase inhibitor disrupts both H- and K-Ras processing and oncogenic signaling.

Thus, we have designed an extremely potent and highly selective FTase inhibitor. FTI-277 inhibited H-Ras processing with concentrations as low as 10 nM, and processing was blocked by more than 95% at 3 μM. The most potent inhibitors previously reported blocked H-Ras processing completely only at 100 μM (28–30). The tremendous increase of potency in intact cells is due to increased hydrophobicity of the central portion of the peptidomimetic. FTI-277 inhibition of FTase resulted in the accumulation of non-farnesylated, GTP-locked H-Ras in the cytoplasm, where it was capable of binding Raf. This sequestration of Raf in the cytoplasm prevented its recruitment to the plasma membrane and subsequent activation. Thus, non-farnesylated cytoplasmic H-Ras could act as a dominant inhibitor by sequestering its downstream effector. Furthermore, FTI-277 was very selective in antagonizing H-Ras-specific signaling. The fact that FTI-277 suppressed only H-RasF but not H-RasGG or Raf oncogenic signaling demonstrates that the suppression is due to inhibition of H-Ras function and not the function of other farnesylated proteins that may be required for H-Ras transformation. Finally, we demonstrated for the first time that an FTase inhibitor can inhibit K-Ras processing and signaling but at much higher doses than required for H-Ras. Since the great majority of human tumors with Ras mutations are of the K-type rather than the H-type, this finding is critical to further development of these agents as anti-cancer drugs.

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